Sexual selection and male reproductive quality in the stalk-eyed fly *Cyrtodiopsis dalmanni*

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ABSTRACT

It is generally assumed that the reproductive success of both males and females is not limited by the availability of ejaculates. However, when male ejaculate production is physiologically constrained, the maintenance of high fertility can be an important determinant of fitness. Under the phenotype-linked fertility hypothesis, females can maximise their fertility by choosing mates on the basis of external phenotypic indicators (exaggerated sexual ornaments or displays) of male reproductive quality.

I test the phenotype-linked fertility hypothesis in the stalk-eyed fly *Cyrtodiopsis dalmanni*, a species characterised by sexual dimorphism in the length of the eyestalks that project laterally from the head (eyespan). I demonstrate that females prefer large eyespan males as mates and exhibit higher fertility when housed with large eyespan males than when housed with small eyespan males. I also show that male eyespan predicts the growth rates and final sizes of the accessory glands and testes of males raised under different levels of nutritional stress. Thus male eyespan is a reliable indicator of male reproductive quality, and female preference for this trait can directly increase female fitness through fertility assurance.

The higher fertility of large eyespan males is associated with the ability to copulate at a higher frequency rather than greater success on a per-mating basis. Using artificial selection experiments and behavioural observations, I provide evidence that male mating frequency is physiologically constrained by the size of the accessory glands. As eyespan reflects male accessory gland size, females can improve their chances of obtaining an ejaculate by choosing mates with large eyespan. Moreover, I show that males allocate larger ejaculates to females that offer a greater number of fertilisation opportunities.

Based on my results, I have proposed a physiological mechanism for the signalling of male mating frequency by male eyespan mediated by circulating levels of juvenile hormone.

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General introduction

1.1 Overview

Many hypotheses have been put forward to explain the evolution of elaborate male sexual ornaments. Although these ornaments can decrease male survival, this cost can be – at least initially – more than compensated for by the reproductive advantage gained when females prefer highly ornamented males as mates. Fisher (1930) first realized that when genetic variance exists in both female preference and male ornamentation, choosy females will produce more grandoffspring than will females that mate at random because the sons of ornamented males enjoy the same reproductive advantage as their fathers. Moreover, non-random mating causes preference alleles to become coupled with alleles for the preferred trait. Thus, the higher mating success of ornamented males accelerates the spread of not only the male trait allele, but also the preference allele, causing the frequency and reproductive advantage of ornamented males to increase each generation. The genetic correlation between preference and trait can cause a 'runaway' process leading to the evolution of increasingly exaggerated ornaments and stronger preferences. This self-reinforcing process eventually reaches a line of equilibrium, where the costs of producing the ornament are exactly balanced by the reproductive advantage associated with female preference; stronger levels of preference can support higher costs of ornamentation (Kirkpatrick & Ryan 1991).

Under Fisher's self-reinforcing process, male ornaments evolve despite being costly. However, under the indicator mechanism, male ornaments evolve precisely because they are costly (Zahavi 1975). If only the highest quality males are capable of producing a costly ornament, then the ornament provides the female with an indicator of male quality. The male ornament indicates a benefit to his mates. This benefit can be heritable, in the form of offspring of high genetic quality, or non-heritable, in the form of material benefits that directly increase female fitness or that of her offspring. Indicator mechanisms have mainly focussed on the possible genetic benefits from

choice of mates with well-developed secondary sex traits, particularly in species where males provide females only with ejaculates. However, sperm and the many accessory substances transferred in the ejaculate are important determinants of female fecundity, fertility, and longevity. Several models suggest female preference for males that provide material benefits can drive the evolution of exaggerated male sexual ornaments (Heywood 1989, Grafen 1990, Iwasa & Pomiankowski 1999). Under these models, the ability of a male to produce an elaborate sexual ornament and his ability to provision his mate(s) with material benefits are both related to his condition.

This introduction is divided into four sections. First, I explain what condition is and - perhaps more importantly - what it is not, by investigating the history of its use in models of sexual selection. Second, I summarize different models of condition-dependent sexual selection to illustrate that female preference for males in good condition can lead to the extreme exaggeration of male ornaments whether the benefits obtained are genetically or environmentally determined. Third, I review the evidence that male ornaments signal reproductive quality, and that females can benefit from choosing to mate with highly ornamented males through increased fertility. Finally, I introduce stalk-eyed flies (Diptera: Diopsidae) as an ideal model system for studying the direct fertility benefits of female preference for exaggerated male secondary sexual characteristics.

1.2 What is condition?

Confusion concerning the application of the term condition to the handicap principle is the result of conflation of many different usages. In this section, I present a history of its usage and provide an appropriate definition for future studies.

Condition has been used for decades in the fisheries and agricultural industries (e.g. Le Cren 1951). This type of condition (hereafter body condition) provides a

measure of fatness and is used as a predictor of productivity (e.g. Dechow *et al.* 2002). Body condition can be estimated using the relationship between body mass and body length (LeCren 1951) or through a scoring system based on palpation of subcutaneous fat (Henneke *et al.* 1983). In contrast, condition was first related to the handicap principle by Andersson (1982) who used it as an occasional synonym for phenotypic quality. Later, Nur & Hasson (1984, p.278) described condition as "a general, long-term property of the organism" that causes differences in "intrinsic viability". They present a model in which male ornament size is a reliable indicator of this trait.

The concept of a condition-dependent handicap is often erroneously attributed to Maynard Smith (e.g. Collins 1993). Maynard Smith (1985) defined three categories of handicaps: the Zahavi handicap, the revealing handicap, and the conditional handicap. In all categories, production of an ornament reduces male viability, which Maynard Smith (1987, p.12) defined as "all components of fitness other than mating success". Under the Zahavi handicap, all males with the ornament allele produce the same ornament regardless of their viability but ornament production imposes a greater cost on low-viability than high viability males. After viability selection, the proportion of high-viability males in the ornamented group will be higher than the proportion of high-viability males in the unornamented group. Consequently, females exhibiting preference for ornamented males are more likely to mate with a high-viability male than are females that mate at random. Under the revealing handicap, all males with the ornament allele produce an ornament, but low-viability males produce lower quality ornaments than do high quality males. Under the conditional handicap, only high-viability males produce an ornament. Low-viability males never produce an ornament regardless of their genotype at the ornament locus. Maynard Smith used the term conditional in the sense of an 'if...then' logic proposition. If a male has high-viability, then he will produce an ornament. If a male has low-viability, then he will not produce

an ornament. The use of conditional propositions is common in game theory (e.g. Maynard Smith 1982).

The confusion surrounding the meaning of condition in the context of the handicap principle began shortly after Maynard Smith's classification of handicap models. Andersson (1986) used a formal model to investigate the conditional handicap described by Maynard Smith (1985). As in Maynard Smith's model, ornament production was conditional on a male having high viability. However, Andersson (1986) termed this type of handicap a 'condition-dependent' ornament and stated that expression of the ornament "increases with the phenotypic condition of the possessor" (Andersson 1986, p.805). Thus, Andersson (1986) conflated Maynard Smith's use of conditional as a logic proposition with the use of condition as a trait related to viability (*sensu* Andersson 1982, Nur & Hasson 1984).

Although Andersson's usage of condition is related to nutrition and the partitioning of resources, he never makes an explicit association between phenotypic condition and body condition. Simmons (1988) later uses the term body condition, but not in the strict sense of a 'measure of fatness'. He demonstrated that male Northern harrier (*Circus cyaneus*) displays were more intense during food-rich years than food-poor years and claimed this as evidence that male display was dependent on body condition (which was not clearly defined). Condition dependence was not explicitly linked to measures of fatness until Møller (1991b, p.147) stated that "a vast literature on the genetics of condition in domestic animals (e.g. Smith *et al.* 1962) suggests that condition generally has a genetic component." Soon after, empirical studies relating male ornament expression to the agricultural concept of body condition (usually measured as the ratio of body mass to body size) began to appear (e.g. Green 1991, Baker 1992). The use of empirical measures of body condition in sexual selection research increased in frequency after Rowe & Houle (1996, p.1416) defined condition

as "the pool from which resources are allocated". For instance, Kotiaho *et al.* (2001, p.686) argued that "residual mass approximates well the resources that should be available for utilization" and therefore can be used as a measure of condition as defined by Rowe & Houle (1996). Today, condition dependence has become synonymous with 'dependent on body condition' (Tomkins *et al.* 2004) alongside the original (and correct) meaning of 'dependent on viability'.

Early models define the expression of costly sexual ornaments as dependent on an individual's fitness, not fatness. It is possible that in some species, in certain environments, body condition is a good estimate of fitness. However, it should never be assumed that fat content (or any other measure of body condition) is a proxy for measuring genetic contribution to future generations. While fat reserves can clearly increase fitness under certain circumstances (e.g. when access to food is limited), the considerable costs of fat storage are often ignored (Witter & Cuthill 1993). There is no reason to assume *a priori* a positive association between fat reserves and fitness, particularly since the need and ability to store fat will likely interact with other fitness components. For instance, subdominant male birds of a variety of species have been shown to have higher fat reserves than dominant males, which might be attributable to the less predictable food supply encountered by subdominants (Cuthill *et al.* 1997, Pravosudov *et al.* 1999). Without detailed knowledge of the relationship between fat reserves or residual mass and viability, body condition should not be used to assess the condition dependence of sexual ornaments.

If, under the handicap principle, condition is not a measure of fat content, how should it be defined? In order for the handicap principle to operate, condition itself must have two fundamental properties:

1) Higher values of condition must confer greater fitness (Cotton *et al.* 2004a).

2) Condition must have an inexhaustible source of variance (Iwasa & Pomiankowski 1994, 1999, Rowe & Houle 1996, Cotton *et al.* 2004a). The source of this variance can be environmental, genetic (e.g. mutation), or both (e.g. host-parasite coevolution).

Like other traits closely related to fitness, condition is expected to exhibit high levels of environmental variance. Price & Schluter (1991) hypothesized that fitness traits are determined by a large number of underlying 'metric' traits. Thus, the environmental variance in fitness traits arises from the environmental variance in each of these metric traits in addition to the environmental variance in the fitness trait itself. Three recent studies (Kruuk *et al.* 2000, Merilä & Sheldon 2000, McCleery *et al.* 2004) have demonstrated strong positive relationships between the environmental variance affecting a trait and the association of a trait with fitness. Therefore, if condition is closely associated with fitness (the first property of condition), it will be characterized by large standing pool of variance that is maintained despite strong selection (the second property of condition).

The maintenance of variance in the genetic components of condition presents a more difficult obstacle because prolonged directional selection arising from female preference will gradually deplete the genetic variance in condition resulting in continuously diminishing benefits to female choice (the lek paradox; Pomiankowski & Møller 1995, Rowe & Houle 1996, Tomkins *et al.* 2004). The solution to this paradox once again lies in the highly polygenic nature of fitness traits. Condition is expected to be influenced by many genes (Price & Schluter 1991) - possibly by every gene in the genome (Andersson 1986). Recent models predict that female preference for condition-dependent ornaments will increase the number of genes that contribute to the phenotypic variance of the male trait (Pomiankowski & Møller 1995, Rowe & Houle 1996). Indeed, sexual traits exhibit higher levels of additive genetic variance than non-

sexual traits (Pomiankowski & Møller 1995). Consequently, condition will represent a large mutational target (Houle *et al.* 1996, Houle 1998), and the corresponding capture of genetic variance will balance the loss resulting from selection at equilibrium. Genetic variation in condition can also be maintained through fluctuating selection, local adaptation, migration (Proulx 2001), and co-evolution (Hamilton & Zuk 1982, Pomiankowski 1987).

The above definition of condition is based on theory, and is not amenable to measurement in practice. As condition is closely related to total fitness, ornament expression is likely to be positively correlated with many different components of fitness. Accordingly, ornament expression will often be associated with phenotypic measures such as body condition. This does not mean that body condition can be equated with the concept of condition required for the handicap principle to operate. Condition likely represents a different suite of traits in different species, and it is possible that different ornaments indicate different components of fitness within a single species. Consequently, there is no single phenotypic trait (other than viability) that can be measured as an index of condition. These limitations do not imply that condition cannot be studied. Three important directions offer immense potential for improving our understanding of condition-dependent traits. First, long-term field studies that demonstrate an association between sexual ornament expression and total fitness are essential (e.g. Reid *et al.* 2005, see Appendix 8.1). Such studies can also help to elucidate the precise components of fitness that make up condition. Second, condition can be experimentally manipulated by altering levels of environmental stress (reviewed in Cotton *et al.* 2004a) or genetic load (Sheridan & Pomiankowski 1997, van Oosterhout *et al.* 2003). Finally, investigation of the genetic and physiological mechanisms underlying ornament expression will eventually provide insight into the nature of condition.

1.3 Theoretical models of condition-dependent sexual selection

So long as condition fulfils the two properties described above, its precise nature has little effect on models of the evolution of condition-dependent sexual selection. In this section, I describe three models of sexual selection to illustrate two important concepts. First, as demonstrated by Grafen (1990), condition dependence can evolve even if neither condition nor male ornament expression has a genetic basis. Second, as demonstrated by Iwasa & Pomiankowski (1994, 1999), the equilibria of condition-dependent models are little affected by the source of variance in condition (i.e. environmental or genetic).

Grafen (1990) describes a haploid population genetic model where a single locus describes advertising rules in males and preference rules in females. The use of a single locus prevents the Fisher process from operating, as it requires independent genetic variation in both traits as well as covariance between them. Male quality (i.e. condition) is environmentally determined, and the single 'rule' allele specifies the relationship between advertising and quality in males. Increasing advertising imposes a greater survival cost on low quality males than on high quality males. Females exhibit higher fecundity when mated to high quality males, but they cannot directly observe male quality. Instead, females base their mating decisions on male advertising, such that female preference is defined as the probability of a female mating with a male of a given advertising level at a particular time of the breeding season. Males can mate repeatedly, but females are limited to a single mating. There are two uninvadable strategies specified by the single locus. First is the non-signalling equilibrium where males advertise at the lowest possible level and females treat all advertising levels the same. Second is the signalling equilibrium where advertising level is a continuous and increasing function of quality, and females pay costs to express preference for high quality over low quality males. The key to understanding Grafen's (1990) model is to

realize that neither male quality nor advertising level (e.g. trait size) is heritable, but the rule that describes the relationship between quality and advertising level (i.e. the degree of condition dependence) is. The existence of males in the population that reduce female fitness to close to zero (e.g. males with extremely low fertility) can result in an unlimited exaggeration of advertising.

Iwasa & Pomiankowski (1994, 1999) provide a quantitative genetic framework for the understanding of condition-dependent signalling. They consider the effect of female preference (p) on the evolution of a costly male signal (s) whose expression is linked to male quality (v). Variation in male quality can be heritable (Iwasa & Pomiankowski 1994) or purely environmentally determined (Iwasa & Pomiankowski 1999); the equilibria of both models take the same form. Female fitness is therefore increased either indirectly (by producing offspring of high genetic quality) or directly (by obtaining material benefits) by mating with high quality males. However, females cannot directly assess male quality. As male ornament size (s) is determined by a condition-dependent component ($t'v$) that is positively correlated with male quality, as well as an arbitrary component that is independent of male quality (t) such that $s = t + t'v$, females can use male ornament size as an indicator of quality. Note that both t and t' are genetic parameters subject to selection, and t' is directly comparable to the rule describing the relationship between male quality and advertising level in Grafen's (1990) model. Female preference is measured as a departure from random mating (defined as $p = 0$) where females with positive preference prefer to mate with males with larger than average ornaments.

Male fitness has three components. First, production of the preferred ornament size increases male mating success by an amount determined by the efficiency of the signal in attracting females (a). Second, exaggerated sexual ornaments impose a cost on the signaller. In the absence of mate choice, natural selection would drive male

ornament size to an optimum ($s = 0$). Departures from this optimum, driven by sexual selection, reduce survival (by an amount c) but the costs are mitigated by male quality: a given deviation in ornament size from the optimum is less deleterious (by an amount k) to high quality males than it is to low quality males. Third, male quality will either directly or indirectly influence male fitness. When male quality is genetic, male quality is positively correlated with survival ($g[v]$). Therefore, genetic male fitness can be expressed as:

$$\ln W_m = a\bar{p}s - \frac{c}{1 + kv} s^2 + g[v]$$

When male quality is environmentally determined, it has no direct effect on a male's fitness. However, by providing their mates with material benefits, high quality males increase the number of offspring they produce. The increase in fitness associated with providing the material benefit will depend on male quality (v) and the relationship between male quality and the material benefits received (h). Environmentally determined male fitness can be expressed as:

$$\ln W_m = a\bar{p}s - \frac{c}{1 + kv} s^2 + hv$$

In the models of Iwasa & Pomiankowski (1994, 1999), female fitness is simpler to define. First, female preference is assumed to be costly, but the magnitude of the cost is not contingent on female quality (i.e. female choice is not condition-dependent). The reduction in survival (b) associated with preference increases exponentially (γ) with the magnitude of the deviation from random mating (p). Second, a female's fitness depends on either her genetic quality or the material benefits received from her mates. If quality is heritable (Iwasa & Pomiankowksi 1994), high quality females have increased survival and female fitness can be expressed as:

$$\ln W_f = -bp^{\gamma} + g[v]$$

When quality is purely environmentally determined (Iwasa & Pomiankowski 1999), a female's fitness is unaffected by her quality but is increased by mating with high quality males through the increase in reproductive success associated with the receipt of material benefits. The magnitude of the benefit is determined by the strength of a female's preference (p), the efficiency of the male signal in attracting females (a), the average condition dependence of male ornament size ($\bar{t}'$), the relationship between male quality and the material benefits received (h), and the environment-induced variance in the quality of her mating partners (σ_v^2). Thus, when male quality is environmentally determined, female fitness can be expressed as:

$$\ln W_f = -bp^\gamma + pa\bar{t}'\sigma_v^2 h$$

Equilibrium occurs when there is no change in female preference over time (i.e. $\Delta p = 0$). At this point, the costs of female preference (determined by b and γ) are exactly balanced by the benefits of mating with high quality males. When male quality is heritable, the costs of preference will be balanced by the increase in offspring fitness obtained by mating with high quality males. The indirect benefits are determined by the efficiency of the male signal in attracting females (a), the average condition dependence of male ornament size ($\bar{t}'$), and the mutational bias that tends to decrease male genetic quality (w). At equilibrium:

$$p^{(\gamma-1)} = \frac{a\bar{t}'w}{\gamma b}$$

When male quality is environmentally determined, the costs of preference will be balanced by the increase in reproductive success associated with the receipt of material benefits. The magnitude of this benefit is determined in the same way as indirect benefits, only it depends on the variance in male phenotypic quality (σ_v^2) and the relationship between male quality and the material benefits received (h). At equilibrium:

$$p^{(\gamma-1)} = \frac{a\bar{t}'\sigma_v^2 h}{\gamma b}$$

Consequently, whether male viability is heritable or environmentally determined, the costs of female preference can be overcome through two mechanisms: (i) increasing the average condition-dependence of the ornament, and (ii) increasing the variance in male quality (through increasing either the mutational or environmental input to the trait).

The equilibria of the genetic and environmental models of Iwasa & Pomiankowski (1994, 1999) on the evolution of costly female preference under the handicap principle are largely interchangeable; one need only alter the source of variance in male quality to convert one model to the other. However, Iwasa & Pomiankowski (1999) – like Grafen (1990) before them – do not explicitly model the cost of providing females with direct material benefits. In models where male quality is genetic, the benefit supplied by males ('good genes') requires no investment of resources. In contrast, the costs of providing material benefits such as parental care or nuptial gifts are almost certainly non-trivial and may generate a trade-off between male attractiveness and the ability to provide material benefits (Kokko 1998). Females might benefit more from mating with unattractive males that invest in material benefits rather than attractive males that do not. Reliable signalling can be maintained by assuming that the relationship between male quality and the production of material benefits is condition-dependent. That is, production of a given benefit must impose a greater cost on low quality males than on high quality males. Inevitably, increasing costs of producing the benefit will reduce the exaggeration of ornament size at equilibrium. If the cost is large enough, it can prevent the evolution of female preference for the male ornament and will favour the evolution of mechanisms for the direct assessment of male quality (Iwasa & Pomiankowski 1999).

1.4 The phenotype-linked fertility hypothesis

The material benefits signalled by male ornaments can take many forms. One intrinsically obvious benefit of mating to females is the receipt of sperm and seminal fluid products crucial to the fertilisation of eggs. Differences in the ability to supply high quality ejaculates between males may favour mate choice in many animals (Andersson 1994). However, remarkably few studies have addressed the possibility of females obtaining direct fertility benefits through preference for highly ornamented males. In this section, I review the available evidence.

Male fertility fulfils the two requirements of condition described above. First, as an important component of reproductive success, higher values of male fertility will be associated with higher fitness. Second, male fertility exhibits high levels of both environmental and genetic variance. For instance, male reproductive quality has been demonstrated to be highly sensitive to nutrition in a wide range of species. Adult diet quality has been shown to be positively correlated with testis mass in the zebra finch *Taeniopygia guttata* (Birkhead *et al.* 1998), accessory gland and testis size in the dung fly *Scatophaga stercoraria* (Foster 1967, Ward & Simmons 1991), accessory gland size in Hawaiian *Drosophila grimshawi* (Droney 1998), spermatophore size in the tiger swallowtail butterfly *Papilio glaucus* (Lederhouse *et al.* 1990), spermatophore number in the water mite *Neumania papillator* (Proctor 1992), and both copulation frequency and inhibition of female remating in the Mediterranean fruit fly *Ceratitis capitata* (Blay & Yuval 1997).

Male fertility is also characterized by high levels of genetic variance. It constitutes a very broad mutational target. Using a *Drosophila melanogaster* library of strains carrying ethylmethanesulfonate-treated homozygous viable second or third chromosomes, Wakimoto *et al.* (2004) estimated that the minimum number of genes essential to male fertility at 500. This estimate ignores any gene where mutations can

affect male fertility but do not result in complete sterility. To compare, only about 3000 *D. melanogaster* genes are essential for viability *sensu stricto* (Koundakjian *et al.* 2004). Mutations resulting in male sterility are approximately 10-20% as frequent as lethal mutations (Wakimoto *et al.* 2004). Consequently, an ornament that reflects the mutational variance in fertility alone could be subject to relatively strong selection at equilibrium.

Trivers (1972, p.167) argued that since "even in males selected for rapid, repeated copulations the ability to do so is not unlimited" females should be selected to avoid males that have depleted their supply of ejaculates. If courtship vigour reflects a male's ejaculate supply, and if secondary sexual structures heighten the appearance of vigorousness, then selection will result in the elaboration of these structures. Unfortunately, the signalling of male reproductive quality through sexual ornament expression was subsequently ignored, largely as a consequence of the simultaneous popularisation of "Bateman's principles" (Trivers 1972).

Bateman's (1948) observations of mating success in *D. melanogaster* have been reduced to three principles (Arnold 1994) of which the most influential is *Bateman's third principle*: the slope of the number of offspring produced regressed on the number of mates is higher in males than females. That is, male reproductive success is more dependent on the number of matings achieved than is female reproductive success. Explanations for this difference usually invoke anisogamy; reproduction is limited by the high costs of producing eggs. Sperm are cheap and plentiful and therefore do not limit male or female reproductive success.

Despite the widespread acceptance and enormous influence of Bateman's principles in sexual selection research, questions about their generality arose soon after Trivers (1972) re-awakened interest in the subject. Dewsbury (1982) argued that although individual sperm are inexpensive to produce, they are not transferred

individually. Ejaculates containing millions of sperm, coupled with accessory materials in the seminal fluid, might be costly to produce. More recent studies have demonstrated costs of ejaculate production, as well as the strategic allocation of a limited number of ejaculates between females (reviewed in Wedell *et al.* 2002; see also Chapter 3). Indeed, even Bateman's (1948) own data show that the difference between males and females is highly variable. In one experimental block, the slope of the regression of offspring number on the number of mates was 17 times higher in males than in females. In the other experimental block, the slope was only 1.6 times higher in males than in females. Birkhead (2000) has attributed the similarity between male and female slopes in the second block to female sperm limitation arising from food-deprived males transferring smaller ejaculates. Sadly, there is no evidence to support this claim.

The phenotype-linked fertility hypothesis (Sheldon 1994) proposes that male sexual ornament expression is positively correlated with male functional fertility. If male sexual ornaments reliably indicate male fertility, females could potentially benefit from mating with highly ornamented males both directly, through increased offspring production, and indirectly, through the production of fertile sons and daughters exhibiting adaptive preference (Pizzari & Birkhead 2002). For the phenotype-linked fertility hypothesis to operate through direct fertility benefits, three criteria must be met. First, male ornament expression must be positively associated with reproductive quality (preferably across different environments). Second, females must prefer to mate with highly ornamented males. Third, female reproductive success must be limited by the availability of ejaculates such that female preference for ornamented males directly increases fertility. Here, I review the evidence for the phenotype-limited fertility hypothesis in birds, fish, mammals, amphibians and insects.

1.4.1 Birds

There is considerable evidence that male reproductive quality is associated with ornament expression in birds. Testis size is positively correlated with the size of a male sexual trait subject to female choice in fowl (*Gallus gallus*, male comb size; Pizzari *et al.* 2004), barn swallows (*Hirundo rustica*, length of male tail feathers; Møller 1994), house sparrows (*Passer domesticus*, male throat badge size; Møller & Erritzøe 1988), and greenfinches (*Carduelis chloris*, male plumage brightness; Merilä & Sheldon 1999). However, the evidence of a relationship between male attractiveness and features of the ejaculate thought to affect fertility is mixed. Although the proportion of motile sperm in an ejaculate is positively correlated with male display rate in the capercaillie (*Tetro urogallus*, Mjelstad 1991), and a similar relationship between sperm velocity and bill colour (lower UV reflectance) has been reported in the mallard (*Anas platyrhynchos*, Peters *et al.* 2004), male sexual traits did not predict features of the ejaculates of the sedge warbler (*Acrocephalus schoenobaenus*, Birkhead *et al.* 1997) or the zebra finch (*Taeniopygia guttata*, Birkhead & Fletcher 1995). Petrie & Williams (1993) found no evidence of an association between male train mass and fertility in the peacock (*Pavo cristatus*).

The observed relationships between male ornament expression and testis size in birds does not necessarily imply that females gain fertility benefits from choosing to mate with attractive males. Most female birds copulate several times during their fertile period (Birkhead *et al.* 1987), often with males other than their mate (extra-pair copulation or EPC), despite a single copulation being sufficient to fertilize an entire clutch in many species (cf. Sax *et al.* 1998). In most cases, females likely engage in EPCs for reasons unrelated to male fertility. For instance, females may be attempting to obtain indirect benefits by seeking out partners of higher genetic quality than their regular mates (Birkhead & Møller 1992). Sperm from the two (or more) males would

mix in the female reproductive tract and be subject to sperm competition. As attractive males are more likely to be involved in EPCs than unattractive males (e.g. Møller 1988, 1992), the frequently observed positive correlation between testis size and ornament expression is likely an adaptation to sperm competition (Møller 1991a). However, it is possible that some females mate multiply in order to maintain high fertility. Females may seek out EPCs as insurance against infertility in their mates. Krokene *et al.* (1998) found that in 3% of blue tit and great tit nests, all offspring were sired by an extra-pair male and attributed the lack of genetic contribution from the cuckolded male to sterility. However, they provided no direct evidence of infertility. Average levels of infertility (eggs that fail to hatch) vary widely between bird species (from 0%-39% of eggs) with an overall average across 64 species of 12% (Morrow *et al.* 2002).

Normally, females store only a small proportion of the sperm transferred in a single ejaculate (<2% Birkhead 1996), suggesting that sperm availability does not limit female reproductive success. However, sperm are gradually lost from the storage organs over time. In species where this rate is high such as the bearded tit *Panurus biarmicus*, females might increase their fertility by mating at regular intervals during the fertile period (Sax *et al.* 1998). Török *et al.* (2003) recently demonstrated that female collared flycatchers (*Ficedula albicollis*) must mate repeatedly during the fertile period to ensure that their eggs are fertilized. Collared flycatchers are unusual because male sperm production capacity is low due to slow replenishment (Michl *et al.* 2002).

1.4.2 Fish

The relationship between male ornament expression and reproductive quality has been investigated in three species of fish. In the Arctic charr (*Salvelinus alpinus*), the intensity of spawning colouration was positively associated with testis mass, ejaculate mass and the concentration of sperm in semen in males kept under laboratory

conditions (Måsvaer *et al.* 2004). However, sperm density was negatively correlated with the intensity of spawning colouration in males collected at spawning sites (Liljedal *et al.* 1999). These contrasting results may arise through highly ornamented males mating more frequently than less ornamented males in the wild. In the roach (*Rutilus rutilus*), highly ornamented males (those with more and larger breeding tubercles) have longer lived sperm than less ornamented males (Kortet *et al.* 2004). Sperm longevity is expected to be an important determinant of fertilisation efficiency in external fertilizers (Snook 2005, but see Gage *et al.* 2004). No relationship between ornamentation and sperm concentration or sperm velocity was detected in the roach (Kortet *et al.* 2004).

Both charr and roach are external fertilisers. Females release unfertilised eggs, sometimes into a nest or shelter but often simply on a rocky area, and males broadcast sperm into the water column. The male ejaculate can become very dilute, particularly in turbulent waters, resulting in low fertilisation success (reviewed in Levitan & Petersen 1995). In the lemon tetra (*Hyphessobrycon pulchripinnis*), another external fertilizer, males fertilize a smaller proportion of available eggs with each consecutive mating (Nakatsuru & Kramer 1982). At high mating frequencies, male reproductive success can become entirely limited by sperm reserves rather than female availability. Female lemon tetra prefer males than have not recently spawned as mating partners (Nakatsuru & Kramer 1982). Thus, females can potentially increase their reproductive success by choosing to mate with a male exhibiting high reproductive quality. Unfortunately, although male lemon tetra do perform a short courtship display, the relationship between courtship and reproductive quality is not known in this species. Furthermore, there is no direct evidence of female preference for highly ornamented males in either the Arctic charr or the roach.

Many studies have examined the relationship between male traits that are subject to female choice and reproductive quality in the guppy (*Poecilia reticulata*). Positive

associations between the number of sperm in a stripped ejaculate (sperm reserves) and both male courtship (sigmoid display) rate (Matthews *et al.* 1997) and coloration (relative area of orange and black spots; Pilastro *et al.* 2002) have been reported. In contrast, Pilastro & Bisazza (1999) found that sperm reserves were negatively correlated with rate of sigmoid display prior to copulation. Nevertheless, as sperm delivery reduced subsequent courtship rate, the male display still signalled reproductive quality. The conflicting results regarding the association between male attractiveness and sperm reserves might arise from variance in this relationship between populations. Pitcher & Evans (2001) reported that in a population of guppies exposed to high predation risk, sigmoid display rate - but not coloration - predicted sperm reserves. In a population exposed to low predation risk, carotenoid colouration - but not melanin colouration or courtship rate - was positively associated with sperm reserves. Finally, when females were artificial inseminated with equal numbers of sperm from two males, the more attractive (colourful) male sired a larger proportion of the resulting offspring than the less attractive male (Evans *et al.* 2003).

Unlike charr and roaches, guppies are live-bearing internal fertilizers. The lack of dilution of the ejaculate and small brood sizes of guppies considerably reduces the potential for sperm limitation. Once-mated female guppies exhibit lower fertility than do multiply-mated females (Evans & Magurran 2000), suggesting that female reproductive success can be limited by ejaculate availability. However, high rates of multiple paternity (Kelly *et al.* 1999) indicate that most females copulate repeatedly in the wild. Consequently, the association between male attractiveness and reproductive quality observed in this species is likely best explained as an adaptation to sperm competition.

1.4.3 Mammals

The phenotype-linked fertility hypothesis has been studied in two mammals: domestic sheep (*Ovis aries*) and red deer (*Cervus elaphus*). Gibson & Jewell (1982) found no association between courtship vigour and semen quality (an index measure based on sperm density, motility, and proportion alive) across four male sheep. Malo *et al.* (2005b) have recently reported a positive association between "antler complexity" (an index of 8 different measurements of antler size and point number) and both relative testis size and sperm velocity (an important determinant of fertility, Malo *et al.* 2005a) in male red deer. However, there is no evidence that any component of antler size or complexity, let alone the specific measure used by Malo *et al.* (2005b), is subject to female choice. Instead, antlers are used in male-male competitions over access to harems of females (Kruuk *et al.* 2002). Consequently, female preference for males in high reproductive quality is unlikely to be responsible for the elaboration of male ornaments in red deer.

In harem-based mammalian mating systems, where the distribution of mating opportunities is highly skewed in favour of a small number of males, dominant males are at risk of sperm depletion (Preston *et al.* 2001). Male fertility is highly variable in wild red deer populations, even when sperm numbers are controlled through artificial insemination. In the red deer population studied by Malo *et al.* (2005b), the percentage of inseminations that resulted in pregnancy varied from 20% to 75% depending on the identity of the donor male. The high mating rates required of dominant males coupled with the low fertilisation success observed in the wild suggest that in some mammal species, female reproductive success might be sperm limited. Further work on the association between male ornaments subject to female choice (such as roaring performance in red deer, McComb 1991) and male reproductive quality is necessary.

1.4.4 Amphibians

Male display is positively correlated with reproductive quality in the Australian frog (*Uperoleia laevigata*; Robertson 1986, 1990). Large males produce calls with lower dominant frequencies than do small males, and they also have disproportionately larger sperm reserves (Robertson 1990). Females prefer to mate with the heaviest male they can support without drowning (approximately 70% of their own body mass). They use the dominant frequency of the male call to assess male size and preferentially approach synthetic songs with frequencies matching males of the appropriate mass (Robertson 1990). *U. laevigata* employs an extremely unusual mechanisms of fertilisation which can require up to 7 hours to complete (Robertson 1986). Females lay each egg in a clutch individually and males must produce a separate ejaculate to fertilise each one. Small males, with high frequency calls, lack sufficient sperm reserves to fertilise the entire clutch of a large female (Robertson 1990). Female spadefoot toads (*Spea multiplicata* and *S. bombifrons*) also have a higher proportion of their eggs fertilized when allowed to mate with a preferred male (one with a high call rate) than when they mate with a non-preferred male (Pfenning 2000).

1.4.5 Insects

Considering the wealth of information on sperm competition in insects, it is surprising that only three studies have addressed the phenotype-linked fertility hypothesis in this group. The best evidence stems from a study of field crickets (*Gryllus lineaticeps*). Female field crickets prefer male calling songs with higher chirp rates and chirp rate is positively correlated with the number of sperm transferred per mating. Wagner & Harper (2003) restricted female mating opportunities to two copulations with a single male and the number of resulting fertilised eggs was positively correlated with male chirp rate, but only when females were fed a low

quality diet. Male ornament expression is also positively associated with reproductive quality in the carrion fly *Prochyliza xanthostoma*. Males with elongate heads are preferred by females and transfer larger ejaculates (Bonduriansky & Rowe 2003). Females of this species rarely remate, suggesting that the fertility of their mates could be of great importance. However, females digest most of the sperm received, hinting that reproductive success is not limited by the availability of sperm (Bonduriansky & Rowe 2003). Finally, males with high courtship rates are preferred by female dung beetles (*Onthophagus taurus*; Kotiaho *et al.* 2001) and male courtship rate is positively genetically correlated with testis weight (Simmons & Kotiaho 2002). There is no evidence that female dung beetles are sperm limited or that mating with preferred males increases fertility.

Maintaining high levels of fertility may represent a widespread problem for female insects. Females of almost all species must mate repeatedly to ensure the fertilisation of their eggs (Ridley 1988). A recent survey of 30 insect species revealed that between 0% and 63% of matings (median = 22%; Garcia-Gonzalez 2004) fail to result in fertilisation. More research on the fertility benefits of mating with highly ornamented males in insects is required.

1.4.6 Summary

Evidence for three criteria outlined above that are required to support the phenotype-linked fertility hypothesis exists for only one species: the Australian frog. However, female preference in this species is not directional. Instead females prefer males with intermediate dominant frequencies and female choice is therefore unlikely to result in the exaggeration of the male display. The many observations of a positive correlation between male ornament expression and reproductive quality are not unexpected, nor are they sufficient to invoke female preference for males of high

reproductive quality as a basis for the evolution of elaborate sexual ornaments. Many male ornaments likely reflect condition (although strong experimental evidence supporting condition dependence is rare; Cotton *et al.* 2004a). Consequently, ornament expression will be positively correlated with most components of fitness including reproductive organ size, ejaculate features, and fertility. Unless an increase in female reproductive success - through higher fertility associated with female preference for highly ornamented males - is demonstrated, positive correlations between ornament expression and male reproductive quality are irrelevant to the issue of adaptive female preference (Johnstone 1995). Moreover, very few studies have examined the relationship between the male ornament and reproductive quality in different environments (see Chapter 2). Positive associations between fitness components under ideal conditions often disappear or even reverse direction in more stressful environments (Messina & Fry 2003). Consequently, the observation of positive correlations under ideal conditions in the laboratory may be misleading. Future studies of the phenotype-linked fertility hypothesis should focus on species exhibiting two characteristics: (i) strong female preference for a male ornament, and (ii) a mating system in which female reproductive success is likely to be limited by ejaculate availability.

1.5 Stalk-eyed flies

Stalk-eyed flies (Diptera: Diopsidae) are an increasingly important model system for the study of sexual selection (Andersson 1994, Wilkinson & Dodson 1997, Wilkinson 2001, Maynard Smith & Harper 2003). In this section, I review the evidence that stalk-eyed flies are ideal subjects for the study of the phenotype-linked fertility hypothesis.

Diopsids are characterised by a form of hypercephaly in which the head capsule is extended laterally in both sexes such that both the eyes and the antennae are located on elongate stalks (Shillito 1940). The distance between the eyes (or the length of the eyestalks) is commonly referred to as eyespan. In many species, males have larger eyespan (both absolute and relative to body size) than do females and sexually dimorphic eyespan has evolved independently at least four times within the family (Baker & Wilkinson 2001). The origin of laterally displaced eyes is likely the result of natural selection for increased visual capacity. The number of ommatidia increases with eyespan, with maximum numbers that are comparable to much larger insects renown for their visual acuity (up to ~2500 in females and ~2600 in males of certain species; de la Motte & Burkhardt 1983). Moreover, in the region of the eye with the highest visual acuity the divergence angles of the ommatidia can be as small as 1.3° resulting in extremely high resolution (Burkhardt & de la Motte 1983). The high number of ommatidia also gives rise to a very large field of binocular vision (135°; de la Motte & Burkhardt 1983). Although binocular vision is common in insects, the large distance between the laterally displaced eyes of Diopsids is predicted to provide a basis for the measurement of the size of a distant object (Burkhardt & de la Motte 1983). That is, stalk-eyed flies likely have stereoscopic vision. Despite these naturally selected advantages, long eyestalks also impose costs including increased susceptibility to damage, reduced aerial performance (Swallow *et al.* 2000), and potential costs arising from the complex neural structures associated with large eyespan (Buschbeck & Hoy 1998).

In South-East Asia, sexually dimorphic Diopsids (*Cyrtodiopsis whitei* and *Cyrtodiopsis dalmanni*) form nocturnal mating aggregations on roothairs that hang underneath stream banks (Burkhardt & de la Motte 1985, Wilkinson & Dodson 1997). Aggregations form at dusk, when males fight for control of harems of females

(Burkhardt & de la Motte 1983); these contests are usually won by the male with the largest eyespan (Panhuis & Wilkinson 1999). Mating occurs at dawn (>90% of copulations occur during this period; Lorch *et al.* 1993), and individual males and females have been reported to mate up to 40 times per day (Burkhardt *et al.* 1994). Mixed-sex aggregations usually comprise 1-4 males and up to 24 females (Lorch *et al.* 1993).

1.5.1 Female preference for male eyespan

An initial naturally selected advantage associated with hypercephaly, however small, would provide a basis for female choice leading to the exaggeration of male eyespan (Cotton *et al.* 2004c). There is considerable evidence that females of the dimorphic species *C. whitei* and *C. dalmanni* prefer males with large eyespan. In laboratory experiments, female *C. whitei* simultaneously presented with large and small 'dummy' males (dead males with artificially lengthened eyespan) preferentially roost with the larger male (Burkhardt & de la Motte 1988). Wilkinson & Reillo (1994) examined female preference in lines of *C. dalmanni* artificially selected for large or small male eyespan to body length ratios. They found that, when simultaneously presented with a large and small eyespan male, females in the lines selected for large male eyespan had similar preference to unselected controls. In contrast, females in the lines selected for small male eyespan chose to roost with the small eyespan male at a significantly higher frequency than control females. The males that were presented to a female were matched for body length, but varied in eyespan by approximately 1mm. Male-male competition was prevented by separating males with a perforated partition through which females could freely pass owing to their smaller size. As selection was restricted to males, the observed change in female preference was attributed to a genetic correlation between male eyespan and female preference.

Using a similar partitioned-male simultaneous choice test, Wilkinson *et al.* (1998) subsequently demonstrated that large eyespan males obtain a higher proportion of copulations than do small males in both *C. dalmanni* and *C. whitei*, but not in their monomorphic congener *C. quinqueguttata*. However, the lack of preference observed in *C. quinqueguttata* may have resulted from the much smaller difference between large and small eyespan males in the monomorphic species than in their dimorphic counterparts.

Further partitioned-male simultaneous choice tests were used to investigate the ability of female *C. dalmanni* to distinguish between males of different eyespans (Hingle *et al.* 2001a). Although no roosting preference was observed in these experiments, females copulated significantly more frequently with large eyespan males when the difference between males was large (mean difference = 3.17mm) but not when the difference was small (mean difference = 1.45mm). When the difference between males was intermediate (mean difference = 2.40mm) females once again copulated more frequently with the large eyespan male, but the strength of preference covaried with female eyespan. The proportion of copulations with the large male was significantly higher for females with large eyespan (>6.00mm) than for females with smaller eyespan (<5.75mm). These results demonstrate that the ability of females to distinguish between males is limited, and fit well with predictions based on models of the Diopsid visual system. A female with a 5.9mm eyespan is expected to be capable of detecting a 1mm difference in male eyespan from a distance of 80mm (assuming mean male eyespan is 8.8mm; de la Motte & Burkhardt 1983). The heightened preference observed in large eyespan females is likely attributable to the positive association between eyespan and visual resolution discussed above. Thus, eyespan fulfils a dual role as both signal in males and receiver in females (de la Motte and Burkhardt, 1983). In subsequent sequential choice experiments in *C. dalmanni*, Hingle *et al.* (2001b)

demonstrated that female preference depended on nutritional status. Females that were fed corn exhibited strong copulation preference for large eyespan males, but mated at random when switched to a sucrose-only diet; the effect was reversible. These results raise the intriguing possibility that female choice might be condition-dependent.

As *C. dalmanni* females lack any obvious rejection response, it can be difficult to tease apart male and female effects on copulation number. Differences in copulation frequency between large and small eyespan males might better reflect male ability than female preference. Indeed, physiological constraints on male copulation frequency are likely important determinants of fitness in *C. dalmanni* because female reproductive success is highly susceptible to ejaculate limitation.

1.5.2 Ejaculate limitation

The offspring of field collected *C. whitei* and *C. dalmanni* exhibit a highly female-biased sex ratio (1♂:1.98♀ in *C. whitei*, 1♂:1.87♀ in *C. dalmanni*; Burkhardt & de la Motte, 1983). Presgraves *et al.* (1997) observed that 29% of field collected *C. whitei* males and between 13-17% of field collected *C. dalmanni* males produce significantly female-biased offspring sex ratios (0-39% male) when mated to virgin females in the laboratory. Breeding experiments revealed that these biased sex ratios are caused by an X-linked selfish genetic element – a phenomenon known as meiotic drive (Presgraves *et al.* 1997). X-linked meiotic drive elements become overrepresented in the gametes of affected males by disrupting the development of sperm lacking the drive element (i.e. Y-bearing sperm). Consequently, the offspring of males with the drive element (SR males) have female-biased sex ratios. In both *C. dalmanni* (Presgraves *et al.* 1997) and *C. whitei* (Wilkinson & Sanchez 2001), the proportion of spermatocyst bundles (groups of 128 maturing spermatids) that are 'degenerate' is negatively correlated with the number of male progeny produced. Thus,

not only do SR males produce female-biased sex ratios, but they also produce fewer sperm (up to 50% less) than do males lacking the drive element (ST males).

Impaired spermatogenesis in SR males can result in both male and female reproductive success being limited by the availability of sperm (Jaenike 1996). Jaenike (1996) proposed that as a meiotic drive element increases in frequency in a population, male mating opportunities will increase both through the increasingly female-biased sex ratio and higher rates of female remating. High female remating rates are expected to evolve for two reasons. First, if females cannot discriminate between SR and ST males, the increasing frequency of mating with SR males will result in higher levels of female sperm limitation. Females will therefore need to remate in order to obtain enough sperm to fertilise their eggs. Second, when ejaculates from an SR male and an ST male mix in the female reproductive tract, SR males obtain few fertilisations. Under these circumstances, SR males sire ~10% of the offspring in *C. whitei* (Wilkinson & Fry 2001); the sperm of SR males, but not of ST males, appear to be rendered inviable by the seminal fluid of rival males (Fry & Wilkinson 2004). When the population sex ratio is female biased, sons increase a female's inclusive fitness more than daughters. By mating frequently, females can minimize the number of their offspring sired by SR males and maintain high proportions of sons.

The presence or absence of a meiotic drive element in male *C. whitei* and *C. dalmanni* does not appear to affect fertility on a per-mating basis. When allowed only a single copulation, females mated with SR males produced similar numbers of offspring as those mated to ST males (*C. dalmanni*, Presgraves *et al.* 1997; *C. whitei*, Fry & Wilkinson 2004). Moreover, the number of sperm stored in the spermathecae of these females was unaffected by male drive genotype (mean ± s.e.: ST = 34.7 ± 2.9, SR = 32.1 ± 3.9; Fry & Wilkinson 2004). However, individual SR males housed with four females for a period of three weeks produced only 73.5% as many offspring as did ST

males treated similarly (*C. whitei*, Wilkinson & Sanchez 2001). This difference is likely attributable to the lower mating frequencies of SR males. In both *C. dalmanni* and *C. whitei*, ST males mate more frequently than SR males when housed with three females and observed for 2.5 hours. SR males mate at 60% the rate of ST males (combined data for both species, Wilkinson *et al.* 2003). Females appear to be incapable of distinguishing SR males from ST males (Wilkinson *et al.* 2003). Consequently, the primary effect of meiotic drive appears to emanate from the inability of SR males to copulate at a high enough frequency to maintain high levels of female fertility.

Even in the absence of meiotic drive, females must mate repeatedly to maintain high levels of fertility. Using a laboratory population of *C. dalmanni* that lacks meiotic drive (the same population described in Chapters 2-6), Baker *et al.* (2001) allowed individual virgin females to mate once, three times, or repeatedly (housed with a single male for the duration of the experiment) with an individual male, and measured female fertility over the next 14 days. Females in all treatments exhibited low fertility. The mean ± s.e. percentage of eggs laid over this period that were fertile was 40% ± 5.5% for once-mated females, 61.9% ± 5.0% for three-times mated females, and only 81.3% ± 3.2% for females allowed continuous access to a male. In contrast, mature females taken directly from the stock cages exhibited >90% fertility. Thus, individual males were incapable of maximizing the fertility of single females even when housed together over long periods of time. Baker *et al.*'s (2001) results are not attributable to genetic incompatibility between males and females, as females mated three-times to the same male had similar fertility to females once-mated to each of three different males.

The low levels of female fertility observed by Baker *et al.* (2001), particularly among once or three-times mated females, might be the result of males transferring small numbers of sperm during copulation. Like most other stalk-eyed flies, *C.*

dalmanni package sperm in secretions from the accessory glands to form spermatophores (Kotrba 1996). Although the number of sperm contained in a spermatophore is unknown, the areas of the spermatophores produced by *C. whitei* and *C. dalmanni* are small, only 4.5% and 6.5% as large (respectively) as the area of the spermatophores produced by the most closely related congener for which data is available: *Teleopsis quadriguttata* (Kotrba 1996). Note that *Teleopsis* and *Cyrtodiopsis* are synonymous (Meier & Baker 2002).

Female-biased sex ratios, high female remating rates, and low fertility arising from meiotic drive and small spermatophore size all likely contribute to male and female reproductive success being limited by ejaculate production. If male attractiveness is positively associated with male fertility, as predicted by the phenotype-linked fertility hypothesis, females would benefit from choosing to roost with large eyespan males. Moreover, as ejaculates become an increasingly valuable resource, males are expected to vary the quantity or quality of their spermatophores with respect to female fecundity and the intensity of sperm competition (Wedell *et al.* 2002, Chapter 3).

The intensity of sperm competition is likely low in *C. dalmanni* and *C. whitei*. Sperm competition arises from the numerical superiority of sperm compared to eggs; sperm from different males compete for access to a scarce resource (Parker 1970). When females have difficulty obtaining enough sperm (or seminal products) to fertilise their eggs, the importance of sperm competition is greatly reduced (Levitan 2004). *C. dalmanni* and *C. whitei* exhibit few adaptations to sperm competition. In contrast to the effects of mating in *D. melanogaster*, copulation does not reduce female longevity (Reguera *et al.* 2004) or receptivity to remating (Grant *et al.* 2002). Nor does it increase female fecundity (Reguera *et al.* 2004). The seminal fluid of rival males does not affect the viability or fertilization efficiency of the sperm of ST males (Fry & Wilkinson

2004). Moreover, each male shares an equal proportion of the offspring when a single female is mated with different ST males on consecutive days indicating a lack of sperm displacement (Wilkinson & Fry 2001). However, there is weak evidence that when females mate with two males in rapid succession (i.e. within 20 minutes), the spermatophore of the first male acts as a mating plug preventing the uptake of sperm and seminal fluid from the second (Lorch *et al.* 1993). By preventing females from obtaining ejaculates, these mating plugs would only exacerbate the ejaculate-limitation of female reproductive success.

1.5.3 Condition dependence of male eyespan

Male eyespan shows heightened condition dependence relative to non-sexual traits in *C. dalmanni*. As condition has a large environmental component (see section 1.2), changing the environment should induce changes in condition. When condition was manipulated by altering the quality or quantity of food available to larvae, male eyespan decreased more in response to increasing levels of stress than did female eyespan, male wing length, or female wing length (David *et al.* 1998, Cotton *et al.* 2004b). These effects were unchanged when body size was included as a covariate in the analyses and are therefore not wholly attributable to correlated changes in body size.

The condition dependence of male eyespan has also been studied in *Diasemopsis aethiopica*, a dimorphic African Diopsid. Knell *et al.* (1999) manipulated condition by raising male and female *D. aethiopica* on either a high quality or a low quality larval diet. In both sexes, flies raised on the low quality diet had smaller eyespans for their body lengths. Females raised on a high quality diet had both larger eyespans and body lengths than did females raised on a low quality diet. In contrast,

males raised on a high quality diet had larger eyespans but similar body lengths compared to males raised on a low quality diet.

Further studies have revealed high levels of genetic variance in the response of male eyespan to larval food stress. Using a full- and half-sib design, David *et al.* (2000) demonstrated that certain genotypes maintain large absolute eyespan across all environments, while others produce smaller absolute eyespan under increasing levels of larval stress (i.e. there was a strong genotype-by-environment interaction). Importantly, most genotypes maintained their ranked positions relative to other genotypes across all environments. That is, the genotype that produced the largest eyespan under low stress produced the largest eyespan under high stress, and the genotype that produced the smallest eyespan under low stress also produced the smallest eyespan under high stress. However, similar effects were observed for non-sexual traits (female eyespan, male and female wing length) suggesting that the observed response in eyespan may have been due to changes in body size. Unfortunately, David *et al.* (2000) attempted to remove the effects of body size by dividing eyespan by thorax length. Such ratio measures do not remove correlations with body size unless trait allometries pass exactly through the origin (Packard & Boardman 1999, Cotton *et al.* 2004a). Cotton (2004) obtained similar results when this experiment was repeated using inbred lines of *C. dalmanni*. By including thorax length as a covariate in their analysis, Cotton (2004) was able to demonstrate that male relative eyespan was much more sensitive to changes in larval diet than were relative measures of female eyespan, female wing length or male wing length. Both David *et al.* (2000) and Cotton (2004) also found that male absolute and relative eyespan exhibited higher genetic variance under high stress levels compared to low stress levels.

Consistent with the assumptions of genetic condition-dependent sexual selection, male eyespan is characterised by high levels of additive genetic variance. The

additive genetic variance in male eyespan in two sexually dimorphic species (*C. whitei* and *C. dalmanni*) is over twenty times greater than that observed for both thorax width in these species and male eyespan in a sexually monomorphic congener (*C. quinqueguttata*). Moreover, additive genetic variance is three times higher for males than females in *C. whitei* and *C. dalmanni* (Wilkinson & Taper 1999). It is therefore not surprising that artificial selection for increased and decreased ratios of male eyespan to body length in *C. dalmanni* produced a rapid bidirectional response (Wilkinson 1993). Approximately 25% of the difference between these artificially selected lines is associated with the X-chromosome (Wolfenbarger & Wilkinson 2001). The X-chromosome of *C. dalmanni* represents approximately 11.9% of the male genome (measured as the length of mitotic chromosomes). If we assume that X-linked genes are dosage compensated through two-fold hyperactivation as in *D. melanogaster* (Meller 2000, see also Appendix 8.2), then the contribution of the X-chromosome to variation is male eyespan is roughly commensurate with its size.

Hypercephaly in general appears to be a complex polygenic trait. Two closely related species of Hawaiian *Drosophila* have been used to study the quantitative genetics of this trait. *D. heteroneura* is highly hypercephalic and sexually dimorphic while *D. silvestris* exhibits a conservative sexually monomorphic head shape. These two species are interfertile. Female *D. heteroneura* prefer to copulate with males with broader heads (Boake *et al.* 1997). Studies of interspecific hybrids have estimated that at least nine genes contribute to head shape differences between these species, compared with only two genes for differences in male face colour and wing spots and a single gene for mesopleural colour (Val 1977, Templeton 1977). The effects of the nine genes contributing to head shape differences are largely additive (Templeton 1977). However, the actual number of genes contributing to head shape differences is almost certainly larger, with every major chromosome contributing to this difference (Lande

1981). The association between hypercephaly and condition may have arisen prior to the evolution of female preference. Cotton *et al.* (2004c) found that both male and female eyespan demonstrate heightened condition dependence relative to wing length in the monomorphic species *Sphyracephala beccarii*. Intriguingly, Cotton *et al.*'s (2004c) results suggest that the reason female preference for hypercephalic males has evolved so frequently is that hypercephaly, by its very nature, reflects male quality.

The nature of the genes contributing to hypercephaly is largely unknown, although genes in the Antennapedia Complex (ANT-C) are strong candidates in *Drosophila*. Mutations at two loci in this complex, *Deformed* and *labial*, produce slightly broadened heads in *D. melanogaster* (reviewed in DeSalle & Carew 1992). To date, studies of gene expression in the eye-antennal disc have not revealed any obvious differences between stalk-eyed flies and *D. melanogaster* (Hurley *et al.* 2001).

1.6 Thesis structure

This thesis comprises five 'results' chapters followed by a discussion and recapitulation of the main findings. The work described in this thesis was carried out under the supervision of Andrew Pomiankowski, Kevin Fowler, and Tracey Chapman, but the design, execution, analysis, and interpretation of all experiments was by the author (with the exception of the data on spermatophore areas included in Chapter 3, which were collected by Claire Grant).

Chapter 2

I report the results of a test of the phenotype-linked fertility hypothesis in *C. dalmanni*. Despite being fixed in size upon emergence from the puparium, male eyespan was a reliable indicator of both accessory gland length and testis length across three different levels of adult nutritional stress. Moreover, females housed with large eyespan males exhibited higher fertility than did females housed with small eyespan

males. The observed positive associations between male eyespan and both fertility and internal reproductive organ size held when the effects of male body size were removed. Thus, females can gain direct fertility benefits from choosing to mate with large eyespan males. This chapter has been submitted for publication to *Proceedings of the Royal Society of London B: Biological Sciences.*

Chapter 3

Two possible non-exclusive mechanisms underlie the association between male eyespan and fertility reported in Chapter 2: (i) large eyespan males induce higher fertility per mating than do small eyespan males, and (ii) large eyespan males are able to mate at higher frequencies than are small eyespan males. In Chapter 3, I describe a test of the first hypothesis. I found no effect of male absolute eyespan on the fertility of once-mated females. This result suggests that the higher fertility of large eyespan males is associated with higher mating frequencies. Unexpectedly, I did find that large eyespan females produced more fertile eggs following a single mating than did small eyespan females. To determine if the higher fertility of large eyespan females was caused by the strategic allocation of ejaculates by males, I re-analysed previously collected data on the sizes of spermatophores transferred by males to large and small eyespan females. Under certain circumstances, males provide large eyespan females with larger spermatophores than they provide to small eyespan females. Thus, males can tailor their limited ejaculates in order to maximize the number of fertilizations obtained. This chapter has been submitted for publication to the *Journal of Evolutionary Biology.*

Chapter 4

The results reported in Chapter 2 indicate that that the increased fertility of large eyespan males arises from their ability to copulate more frequently than small eyespan males. However, it can be difficult to differentiate between the effects of males

and females on mating frequency. Bidirectional artificial selection on male mating frequency in *C. dalmanni* was carried out in order to test if male mating frequency can be limited by male physiological ability rather than the availability of females. Male mating frequency was assayed using a constant, non-limiting, but ecologically realistic number of females and male eyespan was controlled to prevent female choice. I observed a rapid, bidirectional direct response of male mating frequency indicating that males can be limited by their physiological capacity to mate. Moreover, I observed a correlated response to selection in accessory gland length; males selected for high mating frequencies exhibited longer accessory glands than males selected for low mating frequencies. This chapter has been published in the *Journal of Evolutionary Biology* (Rogers *et al.* 2005a).

Chapter 5

The experiment described in Chapter 5 adds to our understanding of the physiological constraints on male mating frequency. I examined the effect of mating on internal reproductive organ size and observed that copulation causes an immediate decrease in male accessory gland size but not in testis size. The pattern of accessory gland size recovery closely mirrored patterns of mating observed in the field. Taken together with the results of Chapter 4, these results indicate that male mating frequency is primarily limited by accessory gland size. This chapter has been accepted for publication in *BMC Evolutionary Biology* (Rogers *et al.* 2005b).

Chapter 6

Having established a relationship between male eyespan, accessory gland size, mating frequency, and fertility - in Chapter 6, I propose that juvenile hormone (JH) could provide a physiological basis for the association between male secondary sexual traits and reproductive quality. JH influences the expression of a male ornament in dung beetles and preliminary evidence suggests it can affect eyespan in stalk-eyed flies.

Here, I report the results of experiments where the juvenile hormone mimic methoprene was topically applied to adult flies. Males treated with methoprene exhibited longer accessory glands and higher mating frequencies than did control males. Moreover, females treated with methoprene had stronger preference for large eyespan males than did control females. Thus, similar physiological mechanisms might influence both ornament expression and female preference in *C. dalmanni*. I discuss the relationship between JH and condition, as well as the evidence that JH influences the development of structural secondary sexual characteristics in insects.

Chapter 7

Chapter 7 provides a summary and brief discussion of the main findings described in this thesis. I also suggest several avenues for future research based on the ideas contained in this thesis.

Chapter 8

Two appendices are included in Chapter 8. These are two articles I helped to write during the course of my PhD studies that are unrelated to my central thesis. Appendix 8.1 (Cotton *et al.* 2005) has been published in *Current Biology*. Appendix 8.2 (Rogers *et al.* 2003) has been published in *BioEssays*.

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2

Male sexual ornament size signals reproductive quality across different environments in a stalk-eyed fly

2.1 Abstract

In species where males only provide ejaculates to their mates, genetic benefits are usually invoked to explain female preference for males with exaggerated sexual ornaments. However, when ejaculates are scarce, females benefit from choosing to mate with highly fertile males. Under the phenotype-linked fertility hypothesis, exaggerated male secondary sexual characteristics can provide a basis for such choice if they accurately reflect male fertility. This hypothesis requires that: (i) the male ornament is subject to female choice, (ii) females mated to highly ornamented males exhibit higher fertility than females mated to less ornamented males, and (iii) the male ornament reliably signals male reproductive quality across different environments. I used the exaggerated eyespan of male stalk-eyed flies (*Cyrtodiopsis dalmanni*) to test this hypothesis. Female preference for large eyespan is well documented in this species. In this chapter, I demonstrate that female fertility is positively correlated with the eyespan of their mates. I then show that although growth and size at maturity of the internal reproductive organs are highly sensitive to adult nutritional stress, male eyespan predicts the growth and final sizes of both the testes and the accessory glands over three different levels of adult nutritional stress. Thus, male eyespan in *C. dalmanni* fulfils all criteria of the phenotype-linked fertility hypothesis.

2.2 Introduction

Female preference for males with exaggerated traits can evolve if the male trait acts as an indicator of male quality (Iwasa & Pomiankowski 1999). When males provide females only with ejaculates, it is often assumed that females benefit from mating with attractive males solely through the production of genetically superior offspring (reviewed in Jennions & Petrie 2000). However, both sperm and the many accessory substances transferred in the ejaculate are important determinants of female fecundity, fertility, and longevity (reviewed in Arnqvist & Nilsson 2000). Consequently, female preference can directly increase fitness even when the male contribution to reproduction is limited to sperm and seminal products.

When female reproductive success is limited by the availability of ejaculates, females will benefit from choosing males with high fertility. As male fertility is determined by internal reproductive traits (e.g. reproductive organ size), females require an external indicator of male reproductive quality in order to assess fertility. Male exaggerated ornaments and displays could provide a basis for choice if they are positively correlated with reproductive quality (the phenotype-linked fertility hypothesis; Sheldon 1994). Positive relationships between male reproductive quality and male signals have been documented in a small number of species. For example, components of male fertility are correlated with plumage brightness in the greenfinch *Carduelis chloris* (Merilä & Sheldon 1999), antler complexity in the red deer *Cervus elaphus* (Malo *et al.* 2005), comb mass in the fowl *Gallus gallus* (Pizzari *et al.* 2004), male display rate in the guppy *Poecilia reticulata* (Matthews *et al.* 1997), courtship rate in the dung beetle *Onthophagus taurus* (Simmons & Kotiaho 2002), and chirp rate in the field cricket *Gryllus lineaticeps* (Wagner & Harper 2003). Positive correlations between male signalling and reproductive quality alone do not constitute convincing evidence that female preference for male sexual signals increases female fertility

(Johnstone 1995). Indeed, as many male signals reflect condition they are predicted to be positively correlated with most components of fitness.

Tests of the phenotype-linked fertility hypothesis must satisfy three criteria. First, the male ornament must be subject to female choice. Second, females that mate with highly ornamented males must exhibit higher fertility than females that mate with less ornamented males; this requires that female reproductive success is frequently limited by fertility. Third, the male ornament must reliably signal male reproductive quality across different environments as trade-offs can cause positive associations between fitness components to disappear or even reverse in direction under stress (Messina & Fry 2003, Greenfield & Rodriguez 2004). Evidence for all three criteria is not currently available for any species.

The stalk-eyed fly *Cyrtodiopsis dalmanni* (Diopsidae) is an ideal model for testing the phenotype-linked fertility hypothesis. In this species, male attractiveness is determined by eyespan, the distance between eyes located on the ends of elongate lateral stalks. A number of experiments have established that females prefer to roost and copulate with males with large eyespan (Wilkinson & Reillo 1994, Hingle *et al.* 2001a,b).

There is ample evidence that fertility assurance is an important component of fitness in *C. dalmanni*. Females store few sperm following a single mating (~ 140 in *C. dalmanni*, unpublished data; ~ 35 in the closely related *C. whitei*, Fry & Wilkinson 2004) probably because male ejaculates are small relative to other Diopsids (Kotrba 1996). As a consequence, multiple copulations are required to achieve and maintain high fertility (Baker *et al.* 2001). This effect is compounded by X-linked meiotic drive found in 13-17% of field collected males (Presgraves *et al.* 1997). This impairs the development of Y-bearing sperm causing low fertility in females mated to drive males (Wilkinson & Sanchez 2001). As females deprived of ejaculates continue to lay

unfertilised eggs (Reguera *et al.* 2004), and a high proportion of males exhibit low fertility, female preference for indicators of male fertility could provide a large reproductive advantage in *C. dalmanni*.

The reproductive success of male *C. dalmanni* is closely associated with the growth and mature size of the internal reproductive organs. The accessory glands and testes are small and immature upon emergence from the puparium and then increase dramatically in size, allowing males to attain sexual maturity only 26 days after eclosion (Baker *et al.* 2003). Accessory gland growth rate is positively correlated with the time required to reach sexual maturity, while the size of the mature accessory glands is both phenotypically (Baker *et al.* 2003) and genetically (Chapter 4) correlated with male mating frequency. The importance of testis size in determining reproductive success in stalk-eyed flies is less well understood, but interspecific studies in other insects suggest that testis size influences male reproductive success under polyandry (Pitnick & Markow 1994, Gage 1994).

In the current chapter, I test the phenotype-linked fertility hypothesis in *C. dalmanni*. Given that female preference for large eyespan males is well documented in this species, I first tested whether females mated to large eyespan males exhibit higher fertility than females mated to small eyespan males. Second, I investigated the reliability of eyespan as a signal of male reproductive quality across different environments. After determining which levels of adult nutritional stress have the greatest effect of the growth of the accessory glands and testes, I examined the relationship between male eyespan and reproductive organ length at these levels of stress.

2.3 Methods

2.3.1 Effects of male eyespan on fertility

Eggs were collected from stock populations of *C. dalmanni* (David *et al.* 1998), and groups of 13 eggs were placed in moist cotton-lined Petri dishes. Larvae were reared under high nutritional stress (0.39g corn per 13 eggs) to generate high variation in male eyespan (Cotton *et al.* 2004a).

Upon eclosion, adult male eyespan and thorax length were measured (see below). Males were assigned to one of two classes (large or small) based on residual eyespan after regression against thorax length using the best fitting line (eyespan = 1.763 (thorax length)$^{1.6649}$). The large and small eyespan classes were defined as the 25% most positive and most negative of the observed residuals, respectively. Intermediate males were discarded.

Adult males were reared individually in 500ml plastic pots with an *ad libitum* diet of corn to sexual maturity (34 days). Each fly was subsequently housed with 8 sexually mature females. After 14 days, these females were discarded and replaced with 8 virgin females. All females used were large, having been reared under low nutritional stress (>2g corn per 13 eggs; Cotton *et al.* 2004a). After 24 hours, males were removed and the eggs laid by each group of 8 females were collected over the next 32 days at 2-3 day intervals. After incubation for four days, eggs were scored as fertile if (i) only the chorion remained, or (ii) eggs remained intact but exhibited signs of development (e.g. segmental striations). The number of eggs laid and the number of fertile eggs were recorded for each group of 8 females. Males that failed to fertilize any eggs were excluded from subsequent analyses.

2.3.2 Effects of adult nutritional stress on reproductive organ growth

Larvae were reared under low nutritional stress (>2g of corn per 13 eggs) to constrain variation in eyespan. At eclosion, male eyespan was measured and flies exhibiting eyespans outside the range of mean ± 1 s.d. were discarded. The remaining males were assigned to one of five diets consisting of *ad libitum* amounts of a homogeneous mixture of corn and sucrose (25% solution containing 3% carboxymethylcellulose, and indigestible starch added to render the viscosity of the sugar solution similar to that of the corn). The five diets were 0% corn, 25% corn, 50% corn, 75% corn, 100% corn (% corn by mass). Males were housed individually in 500ml pots (as above) and provided food every two days. A sample of 11 flies from each diet was dissected every 7 days for 50 days, and the accessory glands and testes of each male were measured (see below). Note that males raised on 100% corn reach sexual maturity after a mean ± s.e. of 26.41 ± 3.07 days while males raised on 0% corn reach sexual maturity after a mean ± s.e. of 29.67 ± 3.09 days (Baker *et al.* 2003). Body length was used to assess male body size in this experiment, and eyespan was treated as a continuous variable.

2.3.3 Effects of male eyespan on reproductive organ growth

Larvae were reared under high nutritional stress (0.39g corn per 13 eggs) to generate variance in eyespan. Adult males were separated into large and small relative eyespan classes (as described above), assigned to one of three diets (0% corn, 25% corn, and 75% corn) and reared as described in the preceding section. A sample of approximately 21 males was dissected every 14 days for 43 days, and the accessory glands and testes of each male were measured. In this experiment, thorax length was preferred to body length used as a more accurate measure of body size while eyespan was treated as a categorical variable.

2.3.4 Morphological measurements

Males were anaesthetised on ice prior to morphological measurements which were conducted using a videomicroscope attached to a computer equipped with NIH Image software. Eyespan was defined as the distance between the outer tips of the eyes. Body length was measured from the anterior tip of the head to the posterior tip of the wings. Thorax length was measured ventrally from the anterior tip of the prothorax along the midline to the joint between the metathoracic legs and the thorax. In the final experiment, two independent measurements revealed that both eyespan ($r = 0.999$, $F_{1, 157} = 5.74x105$, $n_o = 159$) and thorax length ($r = 0.980$, $F_{1, 157} = 7.61x10^3$, $n_o = 159$) were highly repeatable, while classification into large or small eyespan classes was perfectly repeatable. Accessory glands and the uncoiled testes were dissected out in phosphate buffered saline on a glass slide and the length of the line that bisected the middle of each organ was recorded. The means of the two accessory gland measurements and the two testis measurements for each fly were used in the analyses. All traits were measured to the nearest 0.01mm.

2.3.5 Statistical analysis

In the first experiment, the number of fertile eggs produced by each group of 8 females was analysed using a general linear model (GLM). The total number of eggs laid was highly correlated with the number of fertile eggs laid ($r_{73} = 0.727$, $p < 0.0001$) and was therefore included as a covariate in all analyses of male eyespan effects on fertility to control for variance in egg production between groups of females. Either absolute or residual male eyespan (coded as a fixed categorical factor to eliminate any correlation with thorax length) were included to test male eyespan effects on fertility. An ANCOVA of the mean proportion of fertile eggs laid (number of fertile eggs divided by total number of eggs laid) by females mated to large vs. small eyespan

males over time was used to analyse sperm limitation. Data collected in the two days following mating were not included in this analysis as the delay between sperm receipt and its use in fertilisation resulted in low fertility during this period.

GLMs were used to analyse the variation in growth of the testes and accessory glands. Both response variables were log-transformed to normalize their distributions. Initial models included diet, age, eyespan, body length and all possible interactions. Age and diet were coded into all models as fixed ordinal factors. Body size was included as an untransformed continuous variable. Since the flies dissected on day 1 post-eclosion had not been subjected to different diets, they were excluded from the analyses. Models were simplified using stepwise elimination of terms that failed to significantly improve the fit of the model. All final models included all main-effects and the age × diet interaction. The effects of adding other (not significant) interactions to these final models are reported when important to the interpretation. Pairwise comparisons were conducted using Tukey-Kramer HSD tests. The transformed data sets did not significantly deviate from the assumptions of generalised linear modelling and parametric multiple pairwise comparisons. All statistical analyses were conducted using JMP or SPSS 11 statistical analysis programs.

2.4 Results

2.4.1 Effects of male eyespan on fertility

Male absolute eyespan strongly predicted the number of fertile eggs laid over the course of the 32 day collection period ($F_{1,72} = 18.52, p < 0.0001$) when the total number of eggs laid was included as a covariate ($F_{1,72} = 110.37, p < 0.0001$). However, male eyespan did not predict the total number of eggs laid ($F_{1,73} = 0.98, p = 0.3254$).

Estimating the regression coefficient ($b \pm$ s.e.) of the number of fertile eggs on eyespan indicated that females laid 18.16 ± 4.22 more fertile eggs per mm increase in male eyespan (mean eyespan ± s.d. = 7.21mm ± 1.28mm; range: 3.96-9.21mm).

As eyespan is highly correlated with male body size, the above pattern might simply reflect an association between male fertility and general measures of body size, rather than an indicator function of eyespan itself. To discount this possibility, males were classified into one of two groups based on eyespan relative to thorax length (see Methods). Females mated to males with relatively large eyes laid significantly more fertile eggs than those mated to males with relatively small eyes ($F_{1,71} = 6.80, p = 0.0111$; least squares mean ± s.e.: large = 199.8 ± 7.4; small = 172.1 ± 7.5; Fig. 1a). The positive effect of male relative eyespan was independent of the positive relationship between the number of fertile eggs laid and thorax length ($F_{1,71} = 15.96, p = 0.0002$), or the total number of eggs laid ($F_{1,71} = 107.21, p < 0.0001$), included in the model as covariates. As with absolute eyespan, male relative eyespan failed to predict the total number of eggs laid ($F_{1,73} = 0.98, p < 0.3254$).

Females appeared to be sperm limited as only 51.0% of the eggs laid over the 32-day collection period were fertilized. This result might alternatively reflect the failure of some females to mate. The proportion of eggs fertilized declined over time at a rate of -0.02 per day, ($F_{1,18} = 140.56, p < 0.0001$ Fig. 1b) which suggests that ejaculate limitation is an important determinant of fertility.

2.4.2 Effects of adult nutritional stress on reproductive organ growth

Adult nutritional stress inhibited the growth of both the accessory glands and the testes among males raised under low larval stress (accessory glands: $F_{4,346} = 93.14$, $p < 0.0001$; testes: $F_{4,344} = 6.31, p < 0.0001$; Fig 2a,b). Multiple pairwise comparisons (Tukey HSD) revealed that the accessory glands of flies maintained as adults on 0%

corn were significantly smaller than those of flies maintained on any diet and flies maintained on 25% corn exhibited smaller accessory glands than those maintained on higher proportions of corn (all $p < 0.05$; Fig. 2a). Similarly, flies fed 0% corn produced significantly smaller testes than did flies from any other stress regime (all $p < 0.05$), except those maintained on 25% corn ($p > 0.05$; Fig. 2b). No difference in accessory gland or testis length was detected between flies fed 50%, 75%, and 100% corn (all $p > 0.05$).

Both the accessory glands and the testes increased dramatically in length over the first weeks post-eclosion (accessory glands: $F_{6,346} = 319.77, p < 0.0001$; testes: $F_{6,344} = 17.23, p < 0.0001$; Fig 2). However, growth of the male reproductive organs occurred in two stages. During the first two weeks post-eclosion the testes exhibited rapid growth, almost doubling in length from eclosion by day 15 in the low stress groups. Testes reached their final size approximately three weeks post-eclosion. Tukey HSD tests revealed no significant differences in testis length between flies measured at any time between day 22 and day 50 (all $p > 0.05$). In contrast, the accessory glands grew little during the first two weeks post-eclosion. Instead, the increase in accessory gland length occurred primarily between day 15 and day 29 during which time the accessory glands of the low stress groups approximately doubled in length. Accessory gland length reached a plateau between day 43 and day 50 (Tukey HSD, $p > 0.05$). The interaction between age and diet, which had a significant effect on testis length ($F_{24,344} = 2.10, p = 0.0021$) and a marginally significant effect on accessory gland size ($F_{24,346} = 1.54, p = 0.0518$), indicates that flies raised on more stressful diets exhibited slower rates of reproductive organ growth.

Despite the low variance in eyespan exhibited by males in this experiment, absolute eyespan covaried weakly, but positively with both accessory gland length and testis length (accessory glands: $F_{1,346} = 5.47, p = 0.0199$, b ± s.e. = 0.027 ± 0.011;

testes: $F_{1,344} = 4.51, p = 0.0343$, b ± s.e. = 0.021 ± 0.010). In contrast, no significant relationship was detected between body length and the length of either organ (accessory glands: $F_{1,346} = 0.03, p = 0.8701$; testes: $F_{1,344} = 0.00, p = 0.9769$). Absolute eyespan was not correlated with body length ($r_{392} = 0.057, p = 0.2560$).

2.4.3 Effects of male eyespan on reproductive organ growth

To further investigate the association between eyespan and reproductive organ length observed above, I raised males under high larval stress to generate variance in eyespan and thorax length and assigned them to large or small relative eyespan classes (see Methods). The two classes of males differed in eyespan (mean ± s.e.: large = 6.94 mm ± 0.06; small = 6.46 mm ± 0.08; $t_{425} = 4.97, p < 0.0001$) but not thorax length (mean ± s.e.: large = 2.17 mm ± 0.01; small = 2.18 mm ± 0.02; $t_{425} = 0.76, p = 0.4491$). Thus, eyespan class was completely independent of thorax length.

Males with large relative eyespan produced longer reproductive organs than did males with small relative eyespan. Controlling for variance in age, diet, and body size, the accessory glands of large relative eyespan males were significantly longer than those of small relative eyespan males ($F_{1,373} = 7.57, p = 0.0062$, Fig 3a) as were their testes ($F_{1,374} = 9.39, p = 0.0023$, Fig 3b).

Importantly, I found no evidence that the reproductive organs of large eyespan males responded differently to nutritional stress than did those of small eyespan males (eyespan × diet interaction, accessory glands: $F_{2,371} = 0.03, p = 0.9690$; testes: $F_{2,372} = 0.82, p = 0.4401$). Indeed, the difference in accessory gland (Fig. 3a) and testis (Fig. 3b) lengths between large and small eyespan males was remarkably constant across stress levels. On average, accessory gland growth was inhibited by nutritional stress ($F_{2,373} = 3.87, p = 0.0217$) while testis length was not ($F_{2,374} = 1.77, p = 0.1719$). However, the testes did exhibit slower rates of growth under higher levels of nutritional

stress (age × diet interaction: $F_{4,374} = 4.08, p = 0.0030$) as did the accessory glands (age × diet interaction: $F_{4,373} = 8.77, p < 0.0001$). As in the previous experiment, accessory gland and testis length increased with age (accessory glands: $F_{2,373} = 184.44, p < 0.0001$; testes: $F_{2,374} = 20.99, p < 0.0001$), but I found no evidence that the difference in reproductive organ size between large and small eyespan males changed with age (eyespan × age interaction, accessory glands: $F_{2,371} = 0.82, p = 0.4415$; testes: $F_{2,372} = 1.16, p = 0.3155$)

Body size (i.e. thorax length) was a highly significant predictor of accessory gland and testis length (accessory glands: $F_{1,371} = 96.08, p < 0.0001$, b ± s.e = 0.236 ± 0.024; testes: $F_{1,370} = 104.61, p < 0.0001$, b ± s.e = 0.173 ± 0.024). The large regression coefficients indicate that thorax length was a sensitive indicator of reproductive organ size. As with eyespan, the relationship between reproductive organ size and thorax length was unaffected by diet (body size × diet interaction, accessory glands: $F_{2,371} = 0.89, p = 0.4112$; testes: $F_{2,370} = 1.67, p = 0.1906$).

2.5 Discussion

The phenotype-linked fertility hypothesis requires that a male ornament satisfies three criteria. First, the ornament must be subject to female choice. Female preference for large eyespan males is well documented in *C. dalmanni* (Wilkinson & Reillo 1994, Hingle *et al.* 2001a,b). Second, females mated to highly ornamented males must exhibit higher fertility than females mated to less ornamented males. I found that female fertility was positively correlated with the eyespan of their male mating partners. The number of fertile eggs laid increased approximately 10% per millimetre (or ~13% per standard deviation) increase in male absolute eyespan. In addition, large

eyespan males were more fertile than small eyespan males when body size was held constant.

The third requirement of the phenotype-linked fertility hypothesis is that the male ornament reliably signals reproductive quality across different environments. I demonstrated that both absolute eyespan (when variance in body size was minimized) and relative male eyespan predicted reproductive organ length over three different levels of adult nutritional stress. The consistent differences in the lengths of both the accessory glands and the testes between large and small eyespan males across different adult environments provided no evidence of a trade-off between male ornament size and reproductive quality. Thus, male eyespan in *C. dalmanni* meets all three requirements of the phenotype-linked fertility hypothesis.

The observed relationship between male eyespan and fertility could arise through two non-exclusive mechanisms: (i) large eyespan males may be capable of copulating at higher frequencies than small eyespan males, and (ii) large eyespan males may obtain higher numbers of fertilisations per copulation than small eyespan males. The ability of large eyespan males to produce larger reproductive organs than small eyespan males after controlling for body size suggests that both mechanisms are possible. Accessory gland size is both phenotypically and genetically correlated with maximum physiological mating rates in *C. dalmanni* (Baker *et al.* 2003, Chapter 4). The second mechanism might also operate if larger reproductive organs enable the production of larger ejaculates.

In *C. dalmanni*, eyespan and all other external morphological traits are permanently fixed in size shortly after eclosion (Buschbeck *et al.* 2001). This means that eyespan cannot change in response to the adult environment. In contrast, the internal reproductive organs primarily develop after eclosion. I demonstrated that male accessory gland and testis growth are highly sensitive to adult nutritional stress.

Despite being fixed at eclosion, male eyespan was a strong predictor of internal reproductive organ length across different levels of adult nutritional stress. These results suggest that eyespan is a general indicator of male quality consistent with the predictions of the handicap principle (Cotton *et al.* 2004b). If high quality males pay lower marginal costs for the production of both large eyespan and large internal reproductive organs than do low quality males, then one would expect to observe positive correlations – not trade-offs – between male eyespan and reproductive quality across all environments.

The mechanism underlying the observed association between male eyespan and reproductive quality requires further investigation. Previous studies have shown that male eyespan is highly condition-dependent and is determined by male genotype, the larval environment, and their interaction (David *et al.* 1998, 2000; Cotton *et al.* 2004a). It is possible that genes that confer high larval condition (and therefore large eyespan) also confer high adult condition (and therefore high reproductive quality). Alternatively, the level of stress experienced as a larva might induce long lasting physiological changes that affect the allocation of resources to reproduction and survival in adults (e.g. Sørensen & Loeschcke 2001).

2.6 References

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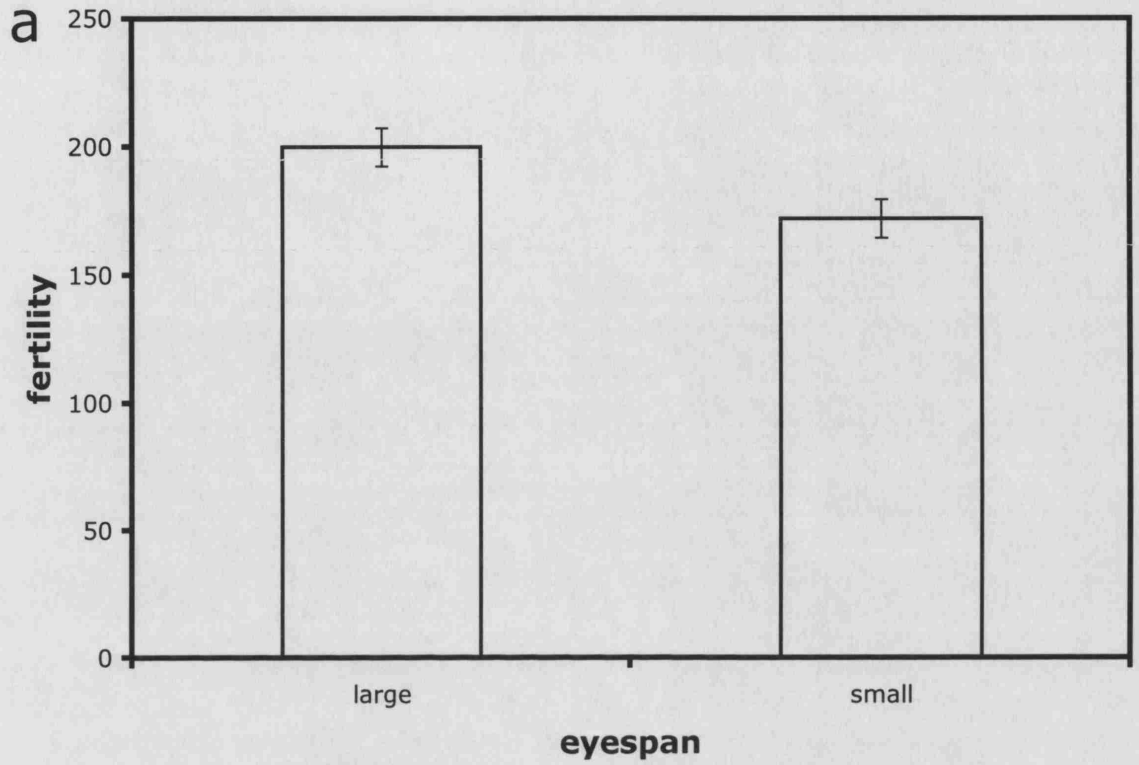
2.7 Figure legends

Fig 2.1 The effect of residual male eyespan on fertility. (a) Least squares means ± s.e. number of fertile eggs laid in 32-day period following mating (at average levels of fecundity) by large and small eyespan males. (b) Decline in the proportion of eggs fertilized by large (filled bars) and small (open bars) eyespan males over time. Bars represent the mean ± s.e. proportion of eggs fertilized on each collection day.

Fig 2.2 The effect of adult nutritional stress on growth of (a) the accessory glands and (b) the testes, in male stalk-eyed flies raised under low larval stress. Organ growth trajectories for flies exposed to one of five different levels of nutritional stress. Data points represent the least squares means ± s.e. for 11 flies (at average value of eyespan). Note that data from day 1 were not included in statistical models.

Fig 2.3 The effect of male eyespan and adult nutritional stress on growth of (a) the accessory glands and (b) the testes, in male stalk-eyed flies raised under high larval stress. Least squares means ± s.e. log reproductive organ length (at average values of thorax length, age) for large (filled bars) and small (open bars) eyespan flies across the three diets.

Figure 2.1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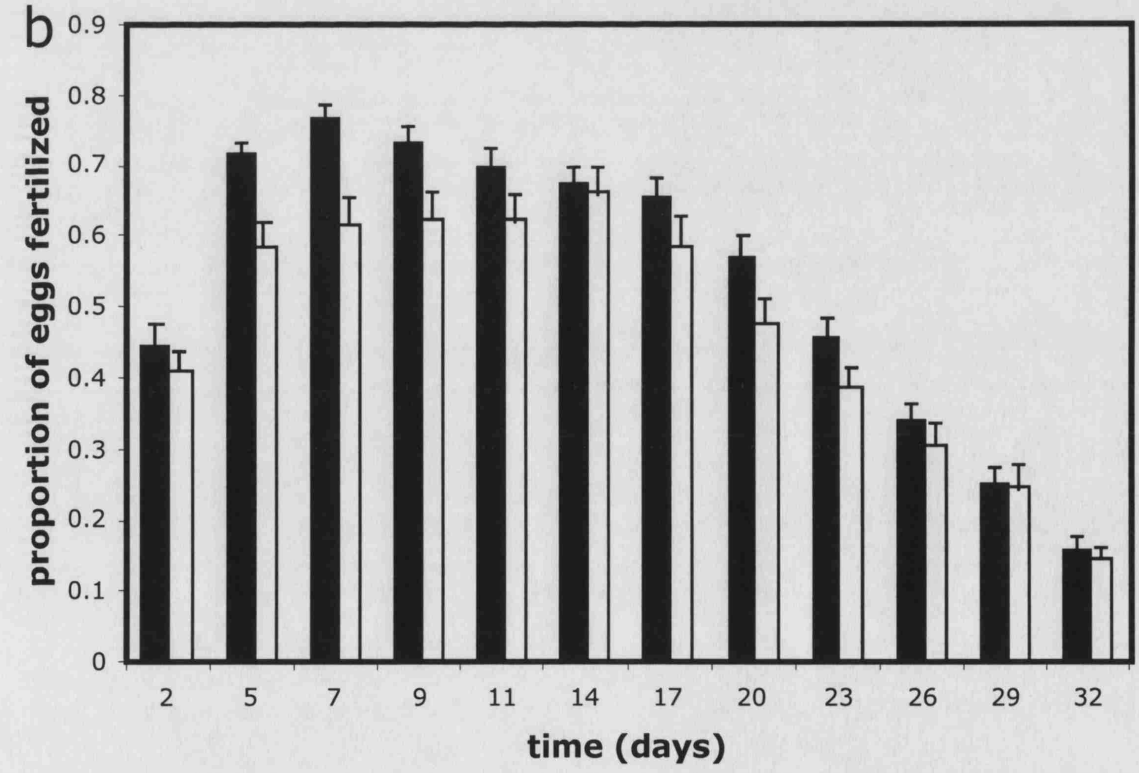
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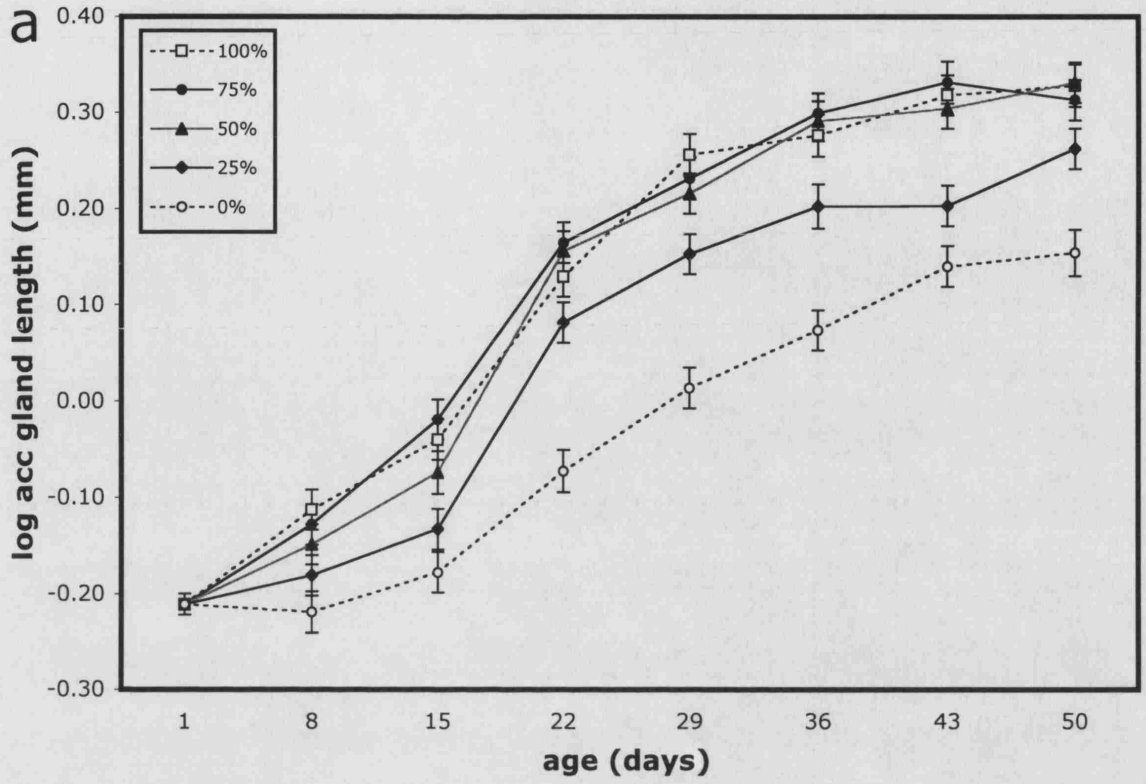
Figure 2.2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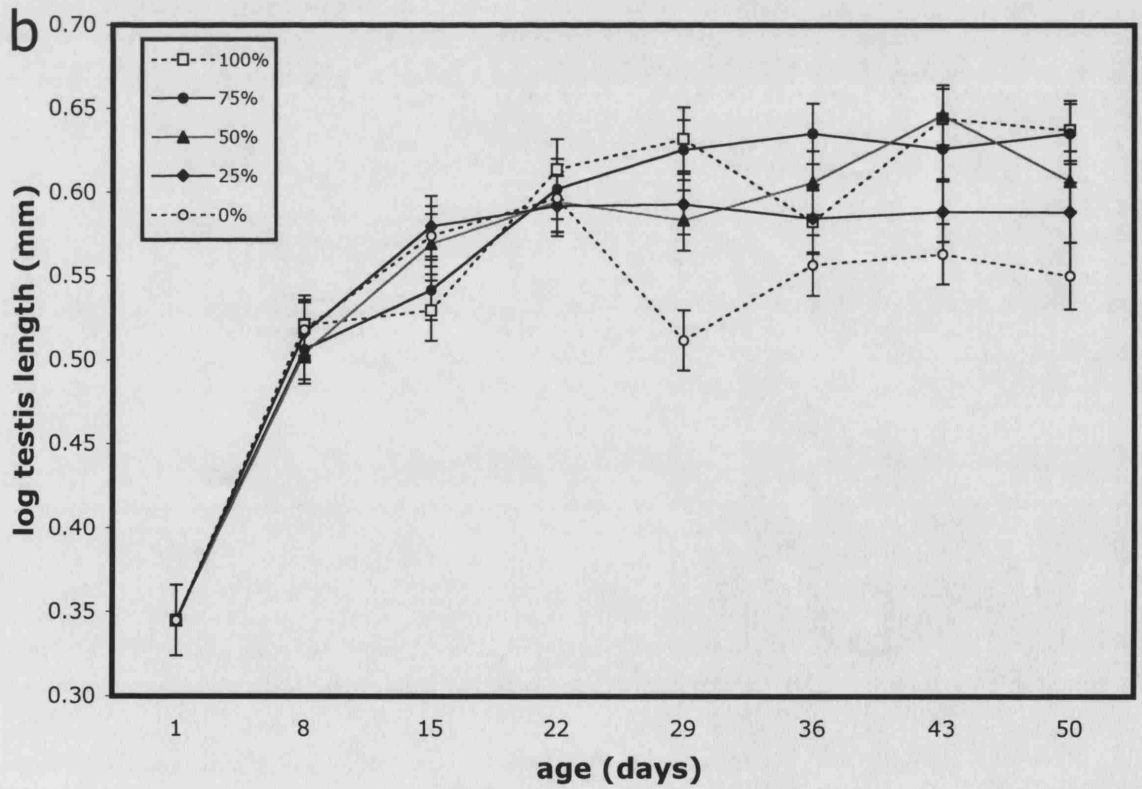
age (days)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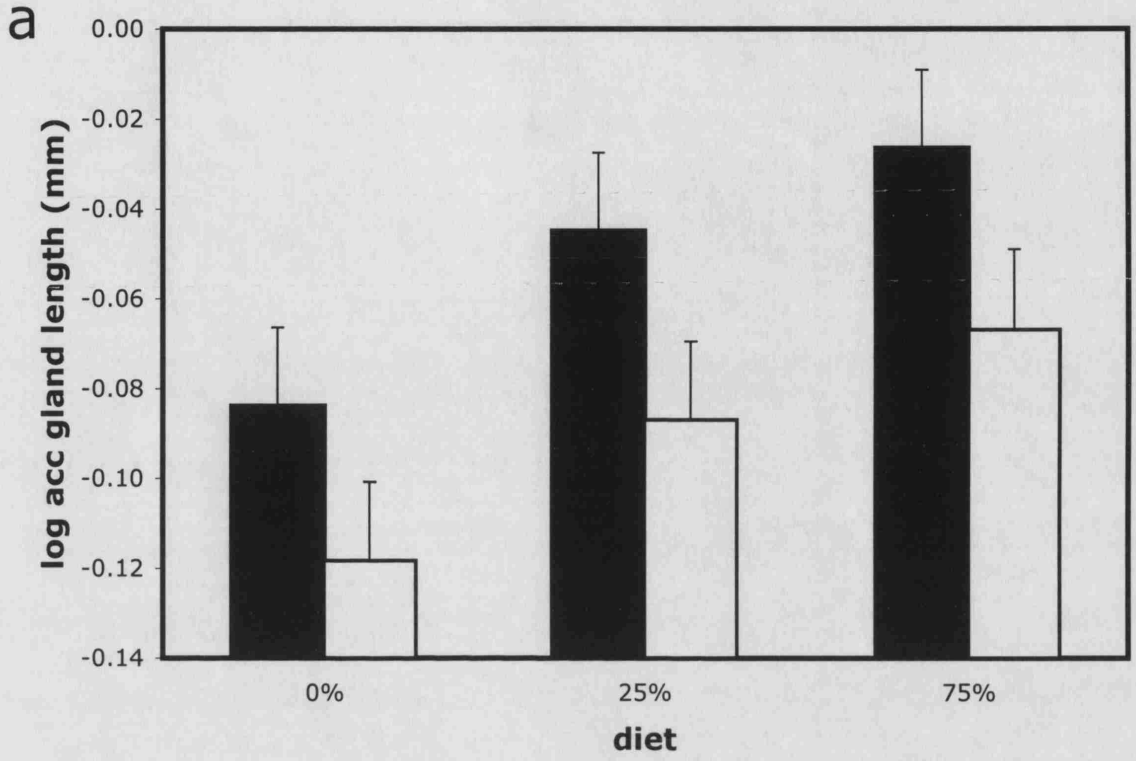
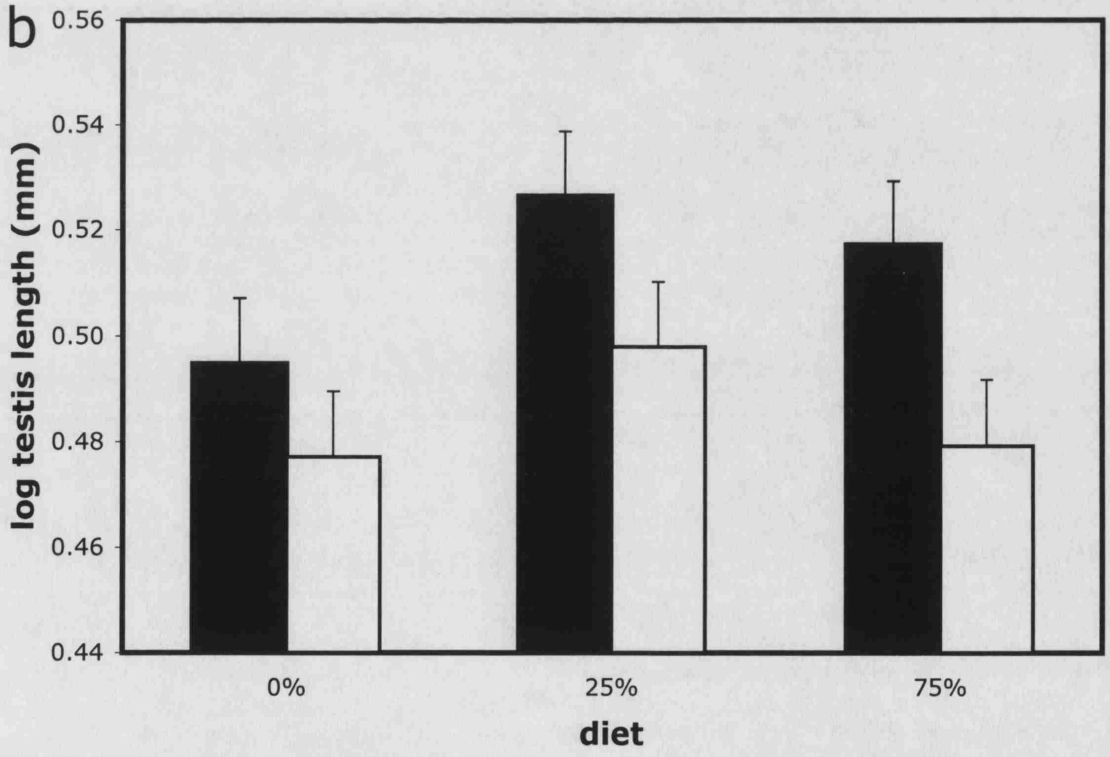
0.50
0.45
0.40
0.35
0.30
100%
75%
50%
25%
0%
1
8
15
22
29
36
43
50
age (days)

Figure 2.3


a
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
log acc gland length (mm)
0%
25%
75%
diet


b
0.56
0.54
0.52
0.50
0.48
0.46
0.44
log testis length (mm)
0%
25%
75%
diet

3

The influence of male and female eyespan on fertility in the stalk-eyed fly *Cyrtodiopsis dalmanni*

3.1 Abstract

The limitation of male reproductive success by the ability to produce ejaculates is expected to elicit behavioural adaptations to increase fertility in both males and females. Males are expected to strategically allocate their ejaculates between females with respect to female reproductive quality and the intensity of sperm competition while females are expected to prefer to mate with highly fertile males. Adult reproductive quality cannot be directly assessed by individuals but it may be reliably signalled by visible phenotypic traits. Hence, I have investigated the potential influence of eyespan, a sexually selected trait, on fertility following a single mating in the sexually dimorphic stalk-eyed fly *Cyrtodiopsis dalmanni*. Although I previously established that females housed with a large eyespan male over 24 hours produced more fertile eggs than did females housed with a small eyespan male, here I failed to detect any association between male eyespan and fertility following a single mating. In contrast, I did find that large eyespan females laid more fertile eggs than did small eyespan females at least in part because males allocated larger spermatophores to large eyespan females. Female eyespan was an accurate predictor of fecundity and large eyespan females laid more than twice as many unfertilized eggs following a single mating as did small eyespan females. The observed difference in fecundity results in higher costs of ejaculate limitation to large eyespan females, and likely underlies the association between female size and the strength of preference previously observed in *C. dalmanni*.

3.2 Introduction

The primary function of the ejaculate is to secure successful fertilisations for the donor male. Beyond the simple delivery of sperm, the ejaculate must fulfil a multitude of roles ranging from facilitating success in sperm competition to the manipulation of female physiology. This requires investment not only in large numbers of sperm but also in large quantities of accessory reproductive products (Moore *et al.* 2004). Consequently even if - as it is widely assumed - sperm production is not very costly, under certain circumstances male ejaculate production can be limited (e.g. Nakatsuru & Kramer 1982, Gage & Cook 1994, Preston *et al.* 2001) or even represent a substantial cost (Dewsbury 1982, Pitnick *et al.* 1995, Olsson *et al.* 1997). When severe, ejaculate limitation can reduce reproductive success and is likely to generate selective pressure for adaptations in both females and males that maximise fertility (reviewed in Wedell *et al.* 2002).

In most insect species, a single copulation typically fails to fertilise all of the eggs produced by a female during her lifetime (Ridley 1988). Two strategies, not mutually exclusive, have been identified that increase female fertility. First, repeated copulation, either with single or multiple males, is a common mechanism of female fertility assurance (Ridley 1988, Arnqvist & Nilsson 2000). For instance, the extremely high female mating frequencies and levels of polyandry (>100 males) observed among queens in certain insect species has been attributed to assurance against ejaculate limitation (Kraus *et al.* 2004). Second, female preference for highly fertile males can maximise reproductive success even when restricted to a single copulation. However, as male fertility is determined by internal factors, direct assessment of male reproductive quality is impossible. Instead, females might use aspects of male phenotype, including sexual ornaments or displays, as indicators of fertility (Sheldon 1994, Iwasa & Pomiankowski 1999). For instance, male fertility correlates with chirp

rate, a sexually selected trait, in the cricket *Gryllus lineaticeps* (Wagner & Harper 2003).

When ejaculate production is limited, males are also expected to exhibit a preference for females of high reproductive quality by strategically allocating larger ejaculates to those females providing the largest fertilisation returns (Wedell *et al.* 2002). The potential number of fertilisations gained from a given mating will depend on both the number of eggs produced by the female, and the level of competition for access to these eggs from other males (Wedell *et al.* 2002). Thus, although highly fecund females offer a high absolute number of fertilisation opportunities, increased investment in high quality females by multiple males normally elevates the intensity of sperm competition, resulting in a decreased number of expected fertilisations per male (Galvani & Johnstone 1998). However, when ejaculate production is severely limited, competition for fertilisations is expected to be low and consequently highly fecund females represent greater fertilisation opportunities. Similar to male fertility, female reproductive quality cannot be directly assessed and males must use phenotypic indicators of fecundity. Female fecundity is closely associated with female body size in many insect species (Honek 1993), and males of many species provide larger ejaculates to larger females (reviewed in Wedell *et al.* 2002).

Stalk-eyed flies are characterized by lateral extension of the head capsule such that the eyes sit on the ends of thin stalks. In *Cyrtodiopsis dalmanni*, the distance between the eyes (eyespan) is significantly greater in males than in females of comparable body size (Baker & Wilkinson 2001). Male eyespan is variable relative to body size (Cotton *et al.* 2004) and is a reliable indicator of internal reproductive organ size (Chapter 2). Females prefer to roost and copulate with males exhibiting large eyespan (Wilkinson & Reillo 1994, Hingle *et al* 2001a,b). Female eyespan is a stronger predictor of body size (Cotton *et al.* 2004) than is male eyespan and could therefore be

used by males to assess female fecundity. *C. dalmanni* females require multiple copulations to achieve high levels of fertility, suggesting that male ejaculate production is limited (Baker *et al.* 2001). The spermatophores of *C. dalmanni* are small relative to other diopsids (Kotrba 1996), and females store few sperm following a single mating (mean ± s.d. = 142.0 ± 63.6; TC, unpublished data).

There is evidence that male eyespan predicts fertility in *C. dalmanni*. When single males were housed with 8 females for 24 hours, partners of large eyespan males exhibited higher fertility than partners of small eyespan males (Chapter 2). However it is not known whether females gained fertility benefits on a per-mating basis by choosing large eyespan males as mates because the number of matings per females was not recorded. Female eyespan may also influence fertility in stalk-eyed flies. If female eyespan indicates fecundity, males should transfer larger ejaculates to large eyespan females resulting in a higher number of fertilised eggs. Here I investigate the effects of male and female eyespan on fertility in *C. dalmanni*. I test whether (i) male eyespan predicts the number of fertile eggs laid following a single mating, (ii) female eyespan indicates fecundity, (iii) female eyespan predicts the number of fertile eggs laid following a single mating, and (iv) males transfer larger ejaculates to large eyespan females than to small eyespan females.

3.3 Methods

3.3.1 Fly stocks and culturing

The flies were from a laboratory population founded in 1993 from individuals captured by AP in Gombak, Malaysia. Large stock populations have been maintained since then in population cages (20x20x30cm) and fed ground corn twice each week.

Flies were kept at a constant temperature of 25°C, with a 12:12 hour dark: light cycle. Artificial dawn was a half-hour period of illumination from a single 60watt bulb, at the start of the light period. Flies for the experiments were obtained by collecting eggs from the stock population cages. Eggs were placed in groups of 13 in Petri dishes lined with moist cotton pads and containing either 2g of ground corn (to produce large flies) or 0.28g of ground corn (to produce small flies).

3.3.2 Effects of male and female eyespan on fertility

At eclosion, individuals were collected and their eyespans were measured using a video camera mounted on a monocular microscope and analyzed using NIH image software (version 1.55). Eyespan was defined as the distance between the outer tips of the eyes of ice-anesthetized flies. Large and small eyespan males and females were raised from eclosion to 6 weeks of age housed in 1.5L plastic pots in single-sex groups of 10 and provided with fresh ground corn and water every 2-3 days. Large and small eyespan females were defined as those with eyespan greater than 6.0 mm and less than 5.0 mm respectively. Large and small eyespan males were defined as those with eyespan greater than 8.3mm and less than 6.2mm, respectively. Other flies with intermediate eyespan were discarded. The experimental flies were raised from eclosion to 6 weeks of age (i.e. to sexual maturity, Baker *et al.* 2003) in 1.5L plastic pots in single-sex groups of 10 and provided with fresh ground corn and water every 2-3 days.

Individual large and small eyespan virgin females were then moved to 500ml plastic pots. Females that failed to lay eggs during the next 7 days were discarded. The following morning, a randomly chosen large or small eyespan male was introduced into each female's pot. Males were allowed to copulate successfully only once with their assigned partner and were then removed. Successful copulations were defined as those greater than 30 seconds in duration. This criterion was chosen to ensure that no female

received more than one spermatophore as copulations shorter than 40 seconds in duration do not result in spermatophore transfer in *C. dalmanni* (DWR unpublished data) or the closely related *C. whitei* (Lorch *et al.* 1993, Fry & Wilkinson 2004).

Eggs were collected from the pots of successfully mated females every 2-3 days and allowed to develop for a further 5 days prior to scoring fertility. An egg was scored as fertile if either only the chorion remained indicating successful hatching or the egg remained intact but exhibited signs of development. Eggs were collected from each female until she failed to produce any fertile eggs for 6 consecutive days. Females that died while still producing fertile eggs were excluded from the analyses, as were females that failed to produce a single fertile egg. Fertility was defined as the total number of fertile eggs collected. Fecundity was defined as the total number of eggs laid during the period in which a female produced fertile eggs.

3.3.3 Effect of female eyespan on spermatophore size

Large and small eyespan females were collected as in the previous experiment, but here they were defined as having eyespan greater than 5.8 mm and less than 5.4 mm, respectively. Any potential effect of male eyespan on spermatophore area was experimentally controlled by using males with low variance in eyespan (mean ± s.d. = 8.73 ± 0.40 mm). Males were mated to females and the size of the ejaculate transferred was estimated by measuring the area of the spermatophore sperm sac deposited inside each female.

Sexually mature males (aged 8-12 weeks) were placed individually in 500 ml pots. On the following morning a single female was introduced to each pot. Half of the males received a large eyespan female and half received a small eyespan female. All flies were observed until a single copulation longer than 40 seconds in duration had taken place after which all the mated females were removed and kept on ice until

dissection. Each male was allowed to mate with a second female of the opposite size class 24 hours later. Those that had previously mated with large eyespan females were tested with small eyespan females and *vice versa*. Males were omitted from the analysis if either spermatophore could not be measured or if they failed to transfer a spermatophore to either female.

To measure spermatophore area, female reproductive tracts were dissected into PBS, and placed on a slide with the ventral sclerite uppermost in a drop of PBS and 0.7µl of Vectashield™ mounting medium with DAPI. Sperm sac area (excluding the tubular neck, see Kotrba 1996) was then measured using NIH image software on images captured from a compound microscope. Each spermatophore was measured either two (n = 144) or three (n = 96) times, and the mean was used in the analysis. The repeatability (Lessels & Boag 1987) of the spermatophore measurements was high ($F_{239,334} = 17.75, p < 0.0001, n_0 = 2.391, r = 0.875$).

3.3.4 Statistical analysis

The length of time a female laid fertile eggs was analyzed using a two-factor ANOVA including male eyespan, female eyespan and their interaction as independent variables. The effects of male and female eyespan on the total number of eggs laid (fecundity) and the number of fertile eggs laid (fertility) were analyzed using general linear models. All main effects (male eyespan, female eyespan, duration of fertile period) were included in the final model, as was the interaction between male and female eyespan, but other less relevant interaction terms were removed from the full model through stepwise elimination of non-significant predictors. A second measure of fertility was obtained by expressing the quotient of the number of fertile eggs laid divided by the total number of eggs laid as a percentage. Fecundity and fertility were normalized using natural logarithm transformations; other variables did not require

transformation. Paired-sample *t*-tests were used to analyze the effect of female eyespan on spermatophore area; the large and small eyespan female that mated with each male constituted each pair.

3.4 Results

3.4.1 Effects of male and female eyespan on fertility

Male eyespan ($F_{1,105} = 0.08, p = 0.7749$), female eyespan ($F_{1,105} = 0.03, p = 0.8685$), and their interaction ($F_{1,105} = 0.03, p = 0.8628$) had no effect on the number of days during which a female produced fertile eggs (overall mean ± s.e. = 20.47 ± 0.59 days, range: 12-32 days).

During this fertile period, large eyespan females were more than twice as fecund as small eyespan females ($F_{1,105} = 69.09, p < 0.0001$). Large eyespan females laid 119.78 ± 5.34 eggs (least squares mean ± s.e.) compared to 52.01 ± 5.44 eggs for small eyespan females when the length of the fertile period, which had a significant effect on the total number of eggs laid ($F_{1,105} = 58.78, p < 0.0001$), was held constant. Male eyespan did not predict female fecundity ($F_{1,105} = 2.06, p = 0.1545$).

Large eyespan females laid more fertile eggs than did small eyespan females. Large eyespan females laid 29.65 ± 1.11 fertile eggs (least squares mean ± s.e.) compared to 20.77 ± 1.11 fertile eggs for small eyespan females (Fig. 1) when the duration of the fertile period was held constant. Females that remained fertile for longer periods laid more fertile eggs ($F_{1,104} = 13.92, p = 0.0003$). Male eyespan did not predict the number of fertile eggs laid ($F_{1,104} = 0.32, p = 0.5749$), nor did large or small eyespan females respond differently to males of different eyespan ($F_{1,104} = 0.03, p = 0.8614$). Despite large eyespan females producing a greater number of fertile eggs, only

a small percentage of their eggs were fertile compared to small eyespan females (t_{107} = 3.80, p = 0.0002; large: 38.86% ± 3.03%; small: 55.20% ± 3.06%).

3.4.2 Effect of female eyespan on spermatophore size

The areas of the spermatophores transferred by a single male to each of his mates (one large and one small eyespan female) were highly positively correlated (r_{115} = 0.5044, $p < 0.0001$). Consequently, the effect of female eyespan on spermatophore area was analysed using a paired t-test to control for variation between males. Males transferred larger spermatophores to large eyespan females than they did to small eyespan females (paired sample t-test, $t_{116} = 2.01$, $p = 0.0468$). The mean ± s.e. area of spermatophores received by large eyespan females was 57.11×10^{-4} mm^2 ± 1.71×10^{-4} mm^2 while the mean ± s.e. area of those received by small eyespan females was 53.61×10^{-4} mm^2 ± 1.79×10^{-4} mm^2. Splitting the data set according to the mating order (Fig. 2) revealed that males only tailored their ejaculates to female size when they mated with a small eyespan female first (paired sample t-test, $t_{52} = 3.23$, $p = 0.0021$; large = 58.27×10^{-4} mm^2 ± 2.84×10^{-4} mm^2, small = 50.20×10^{-4} mm^2 ± 2.32×10^{-4} mm^2) and not when they mated with a large eyespan female first (paired sample t-test, t_{63} = 0.12, $p = 0.9081$; large = 56.15×10^{-4} mm^2 ± 2.29×10^{-4} mm^2, small = 56.43×10^{-4} mm^2 ± 2.44×10^{-4} mm^2).

3.5 Discussion

3.5.1 Effects of male and female eyespan on fertility

I failed to observe any effect of male eyespan on the fertility of females once-mated to virgin males. However, female *C. dalmanni* require multiple copulations in order to fertilise the majority of their eggs (Baker *et al.* 2001). In a previous experiment

where the number of matings was not controlled, I found that females housed with large eyespan males for 24 hours exhibited higher fertility than did females housed with small eyespan males (Chapter 2). Together these results indicate that the higher fertility of large eyespan males is associated with repeated mating.

Male *C. dalmanni* are capable of mating at extremely high frequency (Chapter 4). If males transferred smaller spermatophores during each successive copulation, and the magnitude of this decrease was greater in small eyespan males than in large eyespan males, then females housed with large eyespan males would exhibit higher fertility. However, there is no evidence that male stalk-eyed flies become less fertile over successive matings. The number of sperm stored by a once-mated female is unaffected by the number of copulations her mate engaged in during the previous hour (*C. whitei*; Lorch *et al.* 1993). A more likely explanation for the higher fertility of females housed with large eyespan males is that large eyespan males are simply capable of copulating more frequently than small eyespan males. Provided with a non-limiting number of females, large eyespan (>8.6mm) males mate roughly twice as frequently as small eyespan (<6.4mm) males (Grant 2003). Male mating frequency in *C. dalmanni* is positively correlated with the size of the accessory glands (Baker *et al.* 2003, Chapter 4), and large eyespan males exhibit larger accessory glands than do small eyespan males (Chapter 2).

The behaviour of males carrying a meiotic drive element provides further evidence that fertility is mediated by male mating frequency in stalk-eyed flies. Between 13-17% of field-caught males have an X-linked segregation distorter that impairs the development of Y-bearing sperm (Presgraves *et al.* 1997). These males exhibit similar fertility to standard males following a single mating (Fry & Wilkinson 2004), despite producing significantly fewer functional sperm (Wilkinson & Sanchez 2001). However, drive males only mate 60% as frequently as standard males

(Wilkinson *et al.* 2003) resulting in lower fertility among females housed with drive males over long periods of time, compared to those housed with standard males (Wilkinson & Sanchez 2001).

In contrast to male eyespan, I did find a significant positive relationship between female eyespan and fertility; large eyespan females produced 44% more fertile eggs than did small eyespan females. Multiple factors probably contribute to the observed association between female eyespan and fertility. However, differences in the sizes of the reproductive tracts of large and small eyespan females (including the sperm storage organs and the copulatory bursa) are unlikely to account for the higher fertility of large eyespan females observed in the current study. I allowed females to mate only once, but the capacity of the sperm storage organs greatly exceeds the number of sperm stored following a single mating (*C. whitei*, Lorch *et al.* 1993). Two findings suggest that spermatophore area is not determined by the size of the female copulatory bursa. The areas of the spermatophores transferred to large and small females by individual males were strongly correlated and no effect of female eyespan on spermatophore area was detected when males mated first with large eyespan females.

3.5.2 Effect of female eyespan on spermatophore size

To provide further insight into the higher fertility of large eyespan females, I tested if males are capable of tailoring the sizes of their ejaculates in accordance with the number of fertilisation opportunities offered by their mates. As males cannot directly assess female fecundity, an external indicator is necessary. I found that large eyespan females laid 130% more eggs during their fertile periods than did small eyespan females. Consequently, males could use female eyespan (or any trait correlated with female eyespan) as an indicator of fecundity and adjust the sizes of their spermatophores accordingly.

In agreement with the strategic allocation of ejaculates, I found that males transferred larger spermatophores to large eyespan females than they did to small eyespan females. However, this effect was only significant when males mated with a small eyespan female first. Current evidence is insufficient to explain the importance of mating order on male ejaculate allocation strategy in *C. dalmanni*. Males of this species mate at very high frequency, and it would be informative to examine allocation across a larger number of females. In contrast to fertility, the higher fecundity of large eyespan females is almost certainly not associated with the receipt of larger spermatophores as fecundity is not affected by mating in this species (Reguera *et al.* 2004).

In the current study, spermatophore area was used as a measure of ejaculate quality. I prefer this as a more general measure of male reproductive investment over the more commonly reported value of sperm number, as the latter measure ignores many important components of the ejaculate that contribute to fertility. For instance, *D. melanogaster* males lacking accessory glands (*paired* mutants) are capable of sperm transfer but fail to fertilise their partners' eggs (Xue & Noll 2000). The fertility of *paired* males can be restored by the accessory gland products of spermless males. Accessory gland products are a critical component of the ejaculate and measures of sperm numbers alone may not accurately reflect fertility (Wagner & Harper 2003). Indeed, it is entirely possible that sub-maximal fertility ('sperm' limitation) arises not from a lack of sperm but rather from a deficiency in the other components of the male ejaculate.

3.5.3 Conclusions

I have demonstrated that female eyespan, but not male eyespan, predicts the number of fertile eggs laid following a single mating. Although many factors likely contribute to the higher fertility of large eyespan females, I have provided evidence that

males can adjust the sizes of the their ejaculates to provide large eyespan females with larger spermatophores. Despite the ability of males to tailor their ejaculates, large eyespan females laid many more infertile eggs than did small eyespan females following a single mating. Consequently, to achieve and maintain high fertility and to avoid the costs imposed by laying unfertilised eggs, large eyespan females must mate more often than small eyespan females. As large eyespan males are able to copulate more frequently than are small eyespan males, large eyespan females should be under stronger selection to choose large eyespan males as mating partners. Indeed, female preference for large eyespan males is stronger in larger females and those that are more fecund (Hingle *et al.* 2001a,b). Thus, ejaculate limitation may contribute to the evolution of both strategic ejaculation by males and mate choice by females in *C. dalmanni*.

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3.7 Figure legends

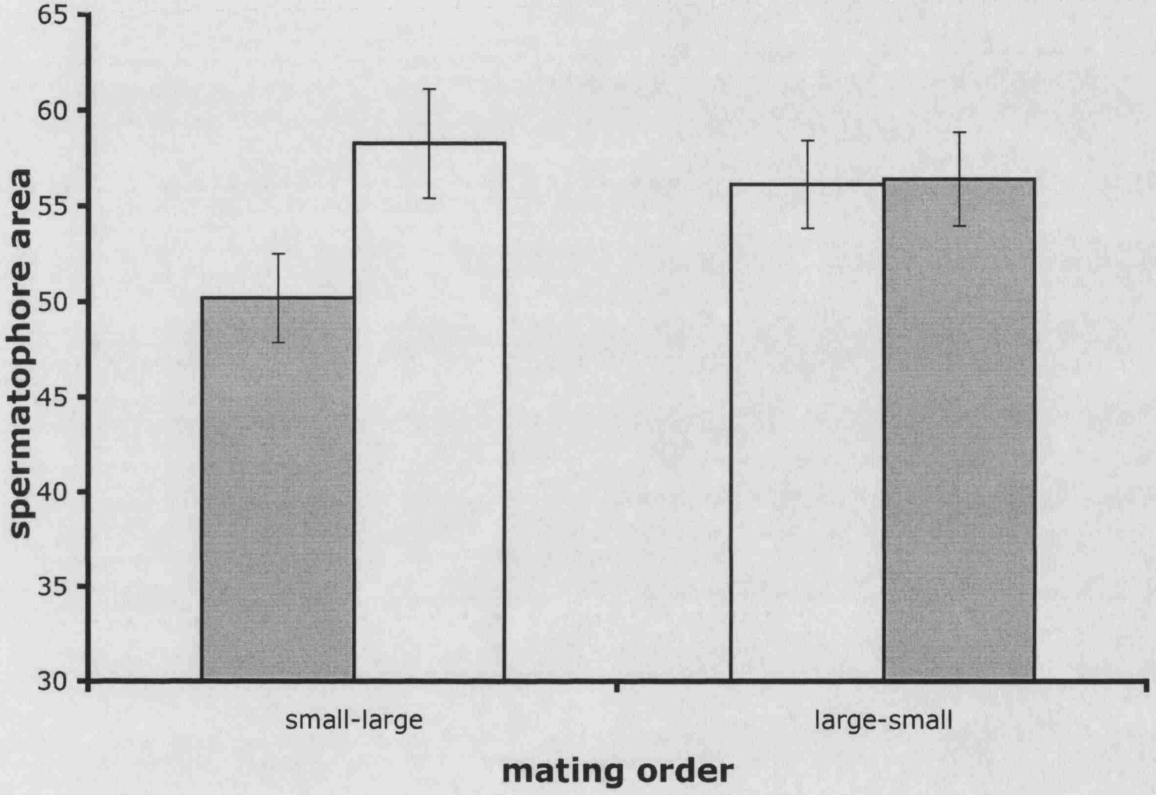
Fig 3.1 Female eyespan, but not male eyespan, predicts fertility after a single copulation. Solid bars: mean fertility of large eyespan males; open bars: mean fertility of small eyespan males. Error bars represent ± 1 s.e.

Fig 3.2. Males tailor ejaculates in accordance with female eyespan. Note that males only adjust their spermatophore areas when mated with a small eyespan female first and a large eyespan female second. Solid bars: mean areas of spermatophores donated to small eyespan females; open bars: mean areas of spermatophores donated to large eyespan females. Error bars represent ± 1 s.e.

Figure 3.1


35
30
25
20
15
10
5
0
number of fertile eggs
large
small
female size

Figure 3.2


65
60
55
50
45
40
35
30
spermatophore area
small-large
large-small
mating order

4

Direct and correlated responses to artificial selection on male mating frequency in the stalk-eyed fly *Cyrtodiopsis dalmanni*

4.1 Abstract

Traditionally it was thought that fitness-related traits such as male mating frequency, with a history of strong directional selection, should have little additive genetic variance and thus respond asymmetrically to bidirectional artificial selection. However recent findings and theory suggest that a balance between selection for increased male mating frequency and opposing selection pressures on physiologically linked traits will cause male mating frequency to have high additive genetic variation and hence respond symmetrically to selection. I tested these hypotheses in the stalk-eyed fly, *Cyrtodiopsis dalmanni*, in which males hold harems comprising many females and so have the opportunity to mate at extremely high frequencies. I subjected male stalk-eyed flies to artificial selection for increased ('high') and decreased ('low') mating frequency in the presence of ecologically realistic, high numbers of females. High line males mated significantly more often than control or low line males. The direct response to selection was approximately symmetric in the high and low lines, revealing high additive genetic variation for, and no significant genetic constraints on increasing, male mating frequency in *C. dalmanni.* In order to investigate trade-offs that might constrain male mating frequency under natural conditions I examined correlated responses to artificial selection. I measured accessory gland length, testis length and eyespan after 7 and 14 generations of selection. High line males had significantly larger accessory glands than low line males. No consistent correlated responses to selection were found in testis length or eyespan. My results suggest that costs associated with the production and maintenance of large accessory glands, although yet to be identified, are likely to be a major constraint on mating frequency in natural populations of *C. dalmanni.*

4.2 Introduction

Male mating frequency is strongly correlated with male reproductive success in many species (Arnold 1994) and is thus expected to be under strong directional selection in natural populations. Traditional life history theory (Falconer & Mackay 1996) predicts that prolonged directional selection on such fitness-related traits will cause the fixation of those alleles with the highest additive effects and the rapid purging of new deleterious mutations, resulting in decreased additive genetic variance (Gustaffson 1986, Jones 1987). Consistent with this idea, Fulker (1966) found relatively low additive genetic variance and strong directional dominance for male mating frequency in *Drosophila melanogaster* and predicted that bidirectional selection on male mating frequency would produce a slow and asymmetric response. In addition, bidirectional artificial selection in *D. melanogaster* for male mating speed, a reproductive trait with a similar genetic architecture to male mating frequency (Casares *et al.* 1993), resulted in decreased, but not increased, responses to selection (Manning 1963, Stamenkovic-Radak *et al.* 1992).

However, there is recent evidence for an alternative view of fitness traits. A survey of experiments by Houle (1992) showed that fitness traits exhibit an order of magnitude more additive genetic variance than morphological traits. One explanation for this discrepancy is that in natural populations, selection for increased values in fitness traits is likely balanced by opposing selection pressures on other physiologically linked traits, i.e. trade-offs between traits (Houle 1998, Merilä & Sheldon 1999). Hence trade-offs allow standing additive genetic variance to be maintained. By overcoming opposing selection pressures, artificial selection can be used to overcome the trade-offs constraining natural selection and expose the underlying genetic variance in fitness

traits. Under this scenario, artificial selection for male mating frequency would be predicted to produce a rapid bidirectional response.

Two major selective pressures are expected to constrain male mating frequency under natural conditions. When males provide no nuptial gifts or parental care, females generally experience smaller gains in reproductive success from mating frequently than do males (Bateman 1948, Arnold & Duvall 1994). Selection for females to avoid the costs of multiple mating, generally higher in females than in males, could then limit the maximum number of mating opportunities available to males. However, in lek- or harem-based mating systems, the mating frequency of attractive or dominant males can become limited not by female receptivity but by the high physiological costs of repeated copulation experienced by males (Dewsbury 1982a,b; Preston *et al.* 2001; Wedell *et al.* 2002). These physiological constraints can be identified by examining the correlated responses to artificial selection on male mating frequency in species where males copulate at high frequency under natural conditions.

I performed artificial selection on male mating frequency in the stalk-eyed fly *Cyrtodiopsis dalmanni*. My aims were (i) to determine the direction and speed of the direct response to selection, and (ii) to reveal the physiological traits that might constrain male mating frequency under natural conditions. This species is ideal for testing these questions because male and female *C. dalmanni* exhibit extremely high mating frequencies, facilitating selection on this trait. Females form nocturnal mating aggregations on root hairs, which males compete to control. As harems controlled by an individual male can include up to 24 females (Lorch *et al.* 1993) and each female is capable of mating six times per hour (Grant 2003), males have extremely high numbers of mating opportunities. Copulation duration in *C.dalmanni* is short (generally lasting less than 60 seconds; Lorch *et al.* 1993) and males are capable of mating up to ten times per hour in the laboratory (Wilkinson *et al.* 1998). Female *C. dalmanni* may mate

often to reduce sperm limitation as they likely store few sperm following a single copulation (~35 in the closely related *Cyrtodiopsis whitei*; Fry & Wilkinson 2004) and require multiple copulations to achieve high fertility (Baker *et al.* 2001). Males transfer small numbers of sperm in spermatophores formed from products of the accessory glands (Kotrba 1996). The small size of *C. dalmanni* spermatophores relative to those of diopsids that mate at lower frequencies (Kotrba 1996) is likely attributable to a male's limited ability to produce sperm or accessory gland products, that is, a cost of spermatophore production. Accessory gland length is phenotypically correlated with male mating frequency in *C. dalmanni* (Baker *et al.* 2003) while testis length is correlated with male mating frequency in other diptera (Blanckenhorn *et al.* 2004). Hence I predict that selection for male mating frequency should produce a correlated response in reproductive organ size.

Lines were selected for increased ('high') and decreased ('low') male mating frequency with two replicates of each selection and control regimes. I adopted two measures to minimize the influence of female receptivity on male mating frequency. First, I monitored the direct response to selection by assaying male mating frequency with high, but ecologically realistic, numbers of randomly-chosen base stock females. Second, larvae were reared under low stress to minimize phenotypic variation in male eyespan (Cotton *et al.* 2004). In *C. dalmanni* male eyespan determines, in part, male attractiveness. Large eyespan males copulate more frequently than small eyespan males under a variety of conditions (Burkhardt & de la Motte 1988; Wilkinson & Reillo 1994, Hingle *et al.* 2001a,b) but females show no preference when presented with a small difference (< 1.45mm) in male eyespan (Hingle *et al.* 2001a). I measured correlated responses to selection in eyespan to control for allometry and to detect any genetic correlation between eyespan and male mating frequency. Furthermore, I assayed

correlated responses to selection in accessory gland length and testis length, after 7 and 14 generations of selection.

4.3 Methods

4.3.1 Base stock and fly handling

The base stock was an outbred laboratory population of the stalk-eyed fly, *C. dalmanni*, collected from Gombak, Malaysia in 1993. The stock was maintained in large cages at high population size (typically more than 200 individuals per cage) and with a 1:1 sex ratio. Flies were fed ground corn medium and kept at 25 °C on a 12h/12h light/dark regime. The lighting regime included a 15-min "dawn" period in which the culture room was illuminated by a single 60-W bulb. All observations of behaviour commenced at the start of this dawn period.

4.3.2 Selection protocol

I produced two replicates each of lines selected for increased ('high' selection regime) or decreased ('low' selection regime) male mating frequency and two unselected 'control' lines from a single base stock population. To obtain flies for the first generation, I reared a synchronously-aged cohort of approximately 800 eggs from the base population under conditions of relaxed competition. After eclosion (approximately 20 days after eggs were laid) batches of 200 adults were placed in population cages and subsequently segregated by sex before reaching sexual maturity. Six weeks after eclosion, 24 males were assayed for mating frequency. To observe matings, individual males were housed with five virgin females in 1500 ml containers that had a central roosting string, a base of moist cotton wool and tissue paper and a

food tray. Males and females were placed in their containers two days prior to the first observation day to acclimatize. Mating frequency was scored as the total number of matings with a duration of more than 40 seconds (the minimum duration required for sperm transfer; Wilkinson & Reillo 1994) achieved in two 90 minute assay periods commencing at artificial dawn on consecutive days.

Of the 24 observed males the eight males with the highest mating frequencies were selected to found replicate 1 of the high selection regime, and the eight males with the lowest mating frequencies founded replicate 1 of the low selection regime. The eight males with intermediate mating frequencies were discarded. A further random sample of eight males was taken from the population cages to found replicate 1 of the control regime. Each male was housed with five females randomly chosen from the base stock population. Eggs laid by the partners of the selected males were collected at two-day intervals, transferred to Petri dishes containing moist cotton pads with *ad libitum* food, and reared until pupariation. Within each replicate line, 40 pupae fathered by each male were pooled within a population cage prior to eclosion. Ensuring equal contributions from selected fathers to the next generation minimized the influence of fecundity selection (Butlin 1993) and prevented selection for low fitness in the low lines. Seven days after the replicate 1 assays, the above procedure was repeated using an independent second random sample of base population flies to found replicate 2 of the high, low and control regimes.

In the second and subsequent generations, the selection protocol was a two-step process in which samples of selection line males were assayed for mating frequency with females from the base population, and were then mated to females of their own line to produce the next generation. Within each selection line the mating frequency of 24 males was measured with five virgin females from the base population. The eight males with the highest or lowest scores were selected to found the next generation of

the high and low lines respectively. Each selected male was mated with five females randomly chosen from his own selection line. Within each control line, the mating frequency of twelve males was measured and eight males were randomly chosen. Forty progeny from each male were collected as pupae and reared to adulthood to produce the next generation of each line. Selection was relaxed in generations 11 and 13 and the mating frequency of control line males was not measured in generation 14.

4.3.3 Correlated responses to selection on male mating frequency

Three male morphological traits were assayed at generation 7 and generation 14 of selection: accessory gland length, testis length and eyespan. All morphological measurements were made using a monocular microscope connected via a video camera to a Macintosh computer with NIH Image (version 1.55).

Males were anaesthetized using ice approximately eight weeks after eclosion. Their accessory glands and testes were dissected out in phosphate buffered saline solution, transferred to a glass slide and uncoiled. Video images of each organ were captured and measured by tracing a midline that bisected the length of the organ. Log-transformed reproductive organ length is highly positively correlated with the log - transformed square root of the longitudinal surface area of each organ (see Chapter 5). Both accessory glands and both testes were measured and the mean of each pair was used in subsequent analyses. Eyespan was defined as the distance between the outer tips of the ommatidia (Hingle *et al.* 2001a).

4.3.4 Statistical analysis

The direct response to selection was analysed by nonparametric methods because the mating frequencies had a highly skewed distribution that could not be normalized by transformation. Consequently, it was necessary to perform independent

analyses of each replicate. The mating frequencies were subjected to one way Kruskal-Wallis analyses of ranks adjusted for ties and unequal sample sizes, followed by multiple pairwise Dunn tests (corrected for unequal sample sizes and ties) if the Kruskal-Wallis test showed a significant difference (Zar 1996).

Each of the correlated responses to selection was normally distributed and the data were analyzed without transformation. The data for accessory gland length and testis length from each replicate were analyzed independently, using generalized linear models (GLMs). For both traits, line and generation were effects-coded as fixed categorical variables. As reproductive organ length can scale with body size, and eyespan is highly correlated with body length (Baker *et al.* 2001), eyespan was included in both models as a continuous variable to account for variation in reproductive organ length associated with differences in body size. GLM analyses were performed using stepwise elimination. If a factor failed to be significant in all models then it was removed from the analysis. Subsequent pairwise comparisons between selection regimes were conducted using Tukey-Kramer HSD tests. The correlated response in eyespan was analyzed using ANOVA with a two-way cross-classification of line (fixed effect) and generation (fixed effect).

I could not exclude the possibility of drift statistically, because the non-normal distribution of the direct response to selection prevented parametric analysis. I have identified responses to selection as those changes that were in the direction predicted *a priori* relative to the controls, that were similar in both replicates and that increased in magnitude over time. All statistical analyses were conducted using JMP software (version 5, SAS Institute Inc.) for the Apple Macintosh. Data are presented throughout as mean ± s.e. unless otherwise specified.

4.4 Results

4.4.1 Direct response to selection on male mating frequency

Observations of mating frequency each generation were used to document the direct response (sample sizes were typically 24 males for each selected line and 12 for the control). There is clear evidence that, in both replicates, selection produced a significant divergence in male mating frequency between selection lines (Fig. 1). I adjusted for variation among generations not due to selection by subtracting the mean rank of the respective control line. Regression through the origin of the mean rank for each selected line against generation then revealed significant direct responses in the high line ($b_1 = 0.69$, $F_{1,11} = 5.53$, $p = 0.0384$) and low line ($b_1 = -1.53$, $F_{1,11} = 48.87$, $p < 0.0001$) of replicate 1, and the high line ($b_1 = 1.714$, $F_{1,11} = 22.61$, $p = 0.0006$) of replicate 2. The low line of replicate 2 showed a negative, but not significant, trend ($b_1 = -0.6196$, $F_{1,11} = 2.96$, $p = 0.1136$).

To provide a more detailed examination of the direct response, I analyzed each generation independently. Later generations were more likely to show significant differences between lines. The high line exhibited significantly higher mating frequencies than the low line at generations 2, 5-9, 12 and 14 in replicate 1 and at generations 4, 6, 8-10, 12, and 14 in replicate 2 (Kruskal-Wallis tests). In general the control lines stayed near to a mating rank of zero (the average rank score for the generation). Moreover, the direct response to selection was bidirectional at particular generations as the high line mated at a significantly higher frequency than the control line in generations 2 and 5 in replicate 1 and generations 8-10, and 12 in replicate 2 while the low line mated at a significantly lower frequency than the control line in generations 8, 9, and 12 in replicate 1 and generations 2, 6, and 12 in replicate 2

(Dunn's tests). Pairwise comparisons were not possible in generation 14 as mating frequencies were not recorded for the control lines.

4.4.2 Correlated responses to selection on male mating frequency

Accessory gland length

Accessory glands were significantly longer in high line males than in low line males across generations in both replicates (Table 1, Fig. 2). The least squares means (mm) ± s.e. adjusted for eyespan and generation were: replicate 1, high line, 2.50 ± 0.03 mm, $n = 98$; control line, 2.36 ± 0.03 mm, $n = 99$; low line, 2.24 ± 0.03 mm, $n = 90$; replicate 2, high line, 2.56 ± 0.03 mm, $n = 73$; control line, 2.56 ± 0.03 mm, $n = 72$; low line, 2.36 ± 0.03 mm, $n = 96$. Pairwise comparisons between lines revealed that low line males had significantly shorter accessory glands than both control and high line males in each replicate. High line males had significantly longer accessory glands than control line males in replicate 1 but not in replicate 2.

In both replicates accessory gland length was shorter in generation 14 than in generation 7 (Fig. 2), as was testis length (Fig. 3) and eyespan (Fig. 4). This response is likely attributable to either inbreeding depression or environmental differences between generations rather than any effect of selection. However, the difference in accessory gland length between high and low lines increased between generation 7 and generation 14 (a significant line x generation interaction), suggesting that continued selection resulted in increased divergence between the lines.

Testis length

No consistent difference was observed between lines in testis length across replicates (Table 2, Fig. 3). The least squares means (mm) ± s.e. adjusted for eyespan

and generation were: replicate 1, high line, 4.50 ± 0.06 mm, $n = 98$; control line, 4.42 ± 0.06 mm, $n = 98$; low line, 4.86 ± 0.06 mm, $n = 90$; replicate 2, high line, 4.67 ± 0.07 mm, $n = 74$; control line, 4.90 ± 0.07 mm, $n = 71$; low line, 4.75 ± 0.06 mm, $n = 95$. Pairwise comparisons between lines revealed that, when averaged across generations, the high line males had significantly longer testes than the low or control line males in replicate 1, and that the control line exhibited significantly longer testes than the high or low lines in replicate 2. Inspection of the data for each generation (Fig. 3) reveals that although the testes of the high line were longer than those of the low and control lines in replicate 1 at generation 7, this difference was not seen in generation 14.

Eyespan

No significant difference was detected between lines in eyespan when averaged across generations in either replicate (Table 3, Fig. 4). The least squares means (mm) ± s.e. averaged across generations were: replicate 1, high line, 8.45 ± 0.04 mm, $n = 99$; control line, 8.46 ± 0.04 mm, $n = 101$; low line, 8.51 ± 0.04 mm, $n = 93$; replicate 2, high line, 8.50 ± 0.04 mm, $n = 82$; control line, 8.44 ± 0.04 mm, $n = 74$; low line, 8.50 ± 0.04 mm, $n = 98$. A significant interaction between line and generation was detected in both replicates. High lines exhibited the largest eyespan in generation 7 but the smallest eyespan in generation 14 in both replicates (Fig. 4).

4.5 Discussion

Artificial selection on male mating frequency over 14 generations produced a rapid, bidirectional direct response; high line (both replicates) and low line (single replicate) males mated significantly more and less frequently, respectively, than did control line males. The observed response suggests that, under natural conditions,

selection on male mating frequency is constrained. Accessory gland size was significantly positively correlated with male mating frequency. This correlated response, and lack of observed response in testis length and eyespan, suggests that the accessory gland length is the primary constraint on male mating frequency in *C. dalmanni.*

Previous artificial selection experiments on dipteran copulatory behavior have generated conflicting results. Only two studies (Manning 1963; Stamenkovic-Radak *et al.* 1992), have selected directly on mating speed, defined as the time to first mating, in male *D. melanogaster*. Both observed responses in the 'slow' direction only. Manning (1961) detected a bidirectional response in male mating speed, but selected simultaneously on both sexes making it difficult to determine the role played by males in the response. Selection for mating speed in both sexes from a mixture of populations of *D. pseudoobscura* resulted in a greater response for fast than slow mating males (Kessler 1969). However, males accounted for only 12% of the variance in overall mating speed. In a similar study using a single population of *D. pseudoobscura*, Spuhler *et al.* (1978) found very little response to selection on mating speed. Mating speed primarily reflects a male's ability to stimulate a female to mate and is strongly influenced by female receptivity. Responses to artificial selection on both sexes are primarily attributable to increased female receptivity. Moreover, the relationship between mating speed and mating frequency remains ambiguous (Arnold & Halliday 1992). In the current study, I have directly selected on mating frequency in males only and, by minimizing the effect of female receptivity on male mating frequency, I restricted selection to a male's physiological ability to copulate repeatedly.

I demonstrated a rapid bidirectional response to selection for male mating frequency. Such responses have been observed in domestic fowl (Dunnington & Siegel 1983; Yang *et al.* 1998), where this trait exhibits high variance associated with male

social dominance. In species where male mating frequency is not limited by female receptivity, high levels of additive genetic variance have likely been maintained primarily through two mechanisms. First, as mating frequency is a highly polygenic trait, directional selection will be countered by the capture of mutational variability across the many underlying loci (Houle 1998). Second, selection for high male mating frequency will be balanced by opposing selection against the associated behavioural and physiological costs.

Analysis of the correlated responses to selection revealed that male accessory gland length is a primary physiological constraint on male mating frequency. Low line males produced significantly smaller accessory glands than the controls in both replicates, and high line males from replicate 1 produced larger accessory glands than controls. Importantly, the difference between high and low lines increased over time indicating divergence in the response to selection. In contrast, no significant correlated response was observed in testis length. These patterns are in line with previous findings in *C. dalmanni* that male mating frequency is phenotypically correlated with accessory gland length, but not testis length (Baker *et al.* 2003). A similar effect of accessory gland size, and lack of effect of testis size, on male mating frequency has been reported for *D. melanogaster* (Bangham *et al.* 2002). Male *D. melanogaster*, after four to five successive matings, fail to fertilize subsequent mates despite possessing adequate supplies of motile sperm in their seminal vesicles. Instead, the contents of the accessory glands of these sexual exhausted males become depleted (Lefevre & Jonsson 1962). Replenishment of depleted accessory gland products requires less than 24-hours in both *D. melanogaster* (Herndon *et al.* 1997) and *C. dalmanni* (Chapter 5), while *de novo* sperm production requires a much longer period of time (10 days in *D. melanogaster*; Lindsley & Tokuyasu 1980). Consequently, the ability to replenish the contents of the accessory glands, rather than the testes, is likely to limit male mating frequency over

short time intervals. In addition to the metabolic costs of replenishing the accessory glands, the development of larger accessory glands delays the onset of sexual maturity in male *C. dalmanni* (Baker *et al.* 2003). Our results indicate that male mating frequency is not limited by sperm production. It remains possible that testis length is an important trait in determining mating frequency over longer time intervals when stores of mature sperm may be depleted.

I detected no correlated response to selection on eyespan. However, this does not necessarily imply that there is no genetic correlation between eyespan and male mating frequency. Eyespan is a condition-dependent trait (David *et al.*, 1998, 2000; Cotton *et al.* 2004) and therefore reflects genetic differences in fitness traits. However, genetic variance in eyespan is masked when flies are not exposed to stress during larval development (David *et al.* 2000). Consequently, as I fed flies on an *ad libitum* diet, my ability to detect genetic differences in eyespan was limited. A physiological link between eyespan and male mating frequency remains a strong possibility.

To conclude, this study shows considerable additive genetic variance for both increased and decreased mating frequency, suggesting that selection favours an optimum that balances the benefits of high reproductive success against the costs of frequent mating. When male mating frequency is not limited by the availability of receptive females, it may become resource-limited. As predicted under anisogamy, the cost of sperm production does not appear to limit male mating frequency in *C. dalmanni*, at least over short periods of time. These results instead indicate that it is the products of the accessory glands that prove limiting in this species.

4.6 References

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Table 4.1 Results of generalised linear model of accessory gland length (mm) of males from a) replicate 1 and b) replicate 2 of the high, low and control lines selected for male mating frequency.

a) Replicate 1

FACTOR	d.f.	Mean square	*F*	*p*
Line	2	1.402	18.57	<0.0001
Generation	1	6.193	82.02	<0.0001
Eyespan	1	1.838	24.35	<0.0001
Line × eyespan	2	0.114	1.50	0.224
Line × generation	2	0.239	3.16	0.044
Error	276	0.076		

b) Replicate 2

FACTOR	d.f.	Mean square	*F*	*p*
Line	2	1.115	15.16	<0.0001
Generation	1	6.579	89.41	<0.0001
Eyespan	1	0.968	13.16	<0.001
Line × eyespan	2	0.366	4.97	0.008
Line × generation	2	0.240	3.25	0.041
Error	232	0.074		

Table 4.2 Results of generalised linear model of testis length (mm) of males from a) replicate 1 and b) replicate 2 of the high, low and control lines selected for male mating frequency.

a) Replicate 1

FACTOR	d.f.	Mean square	*F*	*p*
Line	2	4.583	16.02	<0.0001
Generation	1	27.283	95.39	<0.0001
Eyespan	1	0.207	0.72	0.396
Line × eyespan	2	1.995	6.97	0.001
Line × generation	2	0.786	2.74	0.066
Error	275	0.286		

b) Replicate 2

FACTOR	d.f.	Mean square	*F*	*p*
Line	2	0.982	3.14	0.045
Generation	1	35.753	114.23	<0.0001
Eyespan	1	1.345	4.30	0.039
Line × eyespan	2	0.099	0.31	0.730
Line × generation	2	0.216	0.69	0.503
Error	231	0.313		

Table 4.3 ANOVA of eyespan (mm) of males from a) replicate 1 and b) replicate 2 of the high, low and control lines selected for male mating frequency.

a) Replicate 1

FACTOR	d.f.	Mean square	*F*	*p*
Line	2	0.087	0.53	0.590
Generation	1	5.737	35.16	<0.0001
Line × generation	2	2.918	17.88	<0.0001
Error	287	0.163		

b) Replicate 2

FACTOR	d.f.	Mean square	*F*	*p*
Line	2	0.080	0.57	0.567
Generation	1	1.630	11.59	<0.001
Line × generation	2	1.072	7.62	<0.001
Error	248	0.157		

4.7 Figure Legends

Fig 4.1 Direct responses to bidirectional selection on male mating frequency. The direct response is shown as the standardised mating rank of each selection line plotted against generation of selection for a) replicate 1 and b) replicate 2. Selection line: solid triangles, high; open circles, control; closed squares, low. Within each generation, standardised mating ranks for each line were calculated as: $[R_L - R_G] / s_G$ where R_L denotes the mean rank of a selection line, R_G denotes the mean rank of the pooled selection lines and s_G denotes the standard deviation of ranks for the pooled selection lines.

Fig 4.2 Correlated response of accessory gland length to selection on male mating frequency. Mean accessory gland length (mm) $\pm$ s.e. measured at generations 7 and 14 of selection for a) replicate 1 and b) replicate 2. Selection line: solid triangles, high; open circles, control; closed squares, low.

Fig 4.3 Correlated response of testis length to selection on male mating frequency. Mean testis length (mm) $\pm$ s.e. measured at generations 7 and 14 of selection for a) replicate 1 and b) replicate 2. Selection line: solid triangles, high; open circles, control; closed squares, low.

Fig 4.4 Correlated response of male eyespan to selection on male mating frequency. Mean eyespan (mm) $\pm$ s.e. measured at generations 7 and 14 of selection for a) replicate 1 and b) replicate 2. Selection line: solid triangles, high; open circles, control; closed squares, low.

Figure 4.1

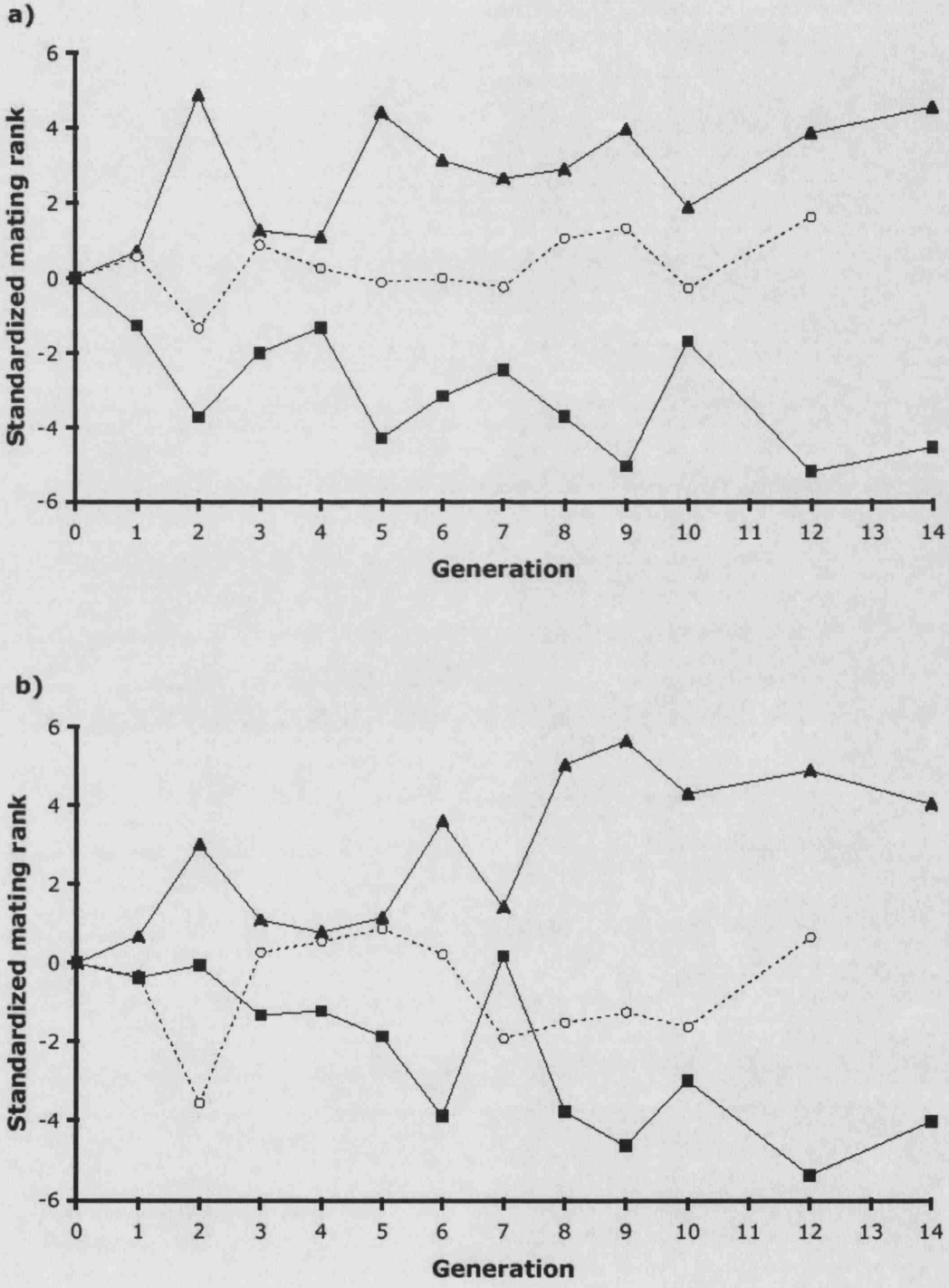

a)
Standardized mating rank
6
4
2
0
-2
-4
-6
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Generation
b)
Standardized mating rank
6
4
2
0
-2
-4
-6
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Generation

Figure 4.2

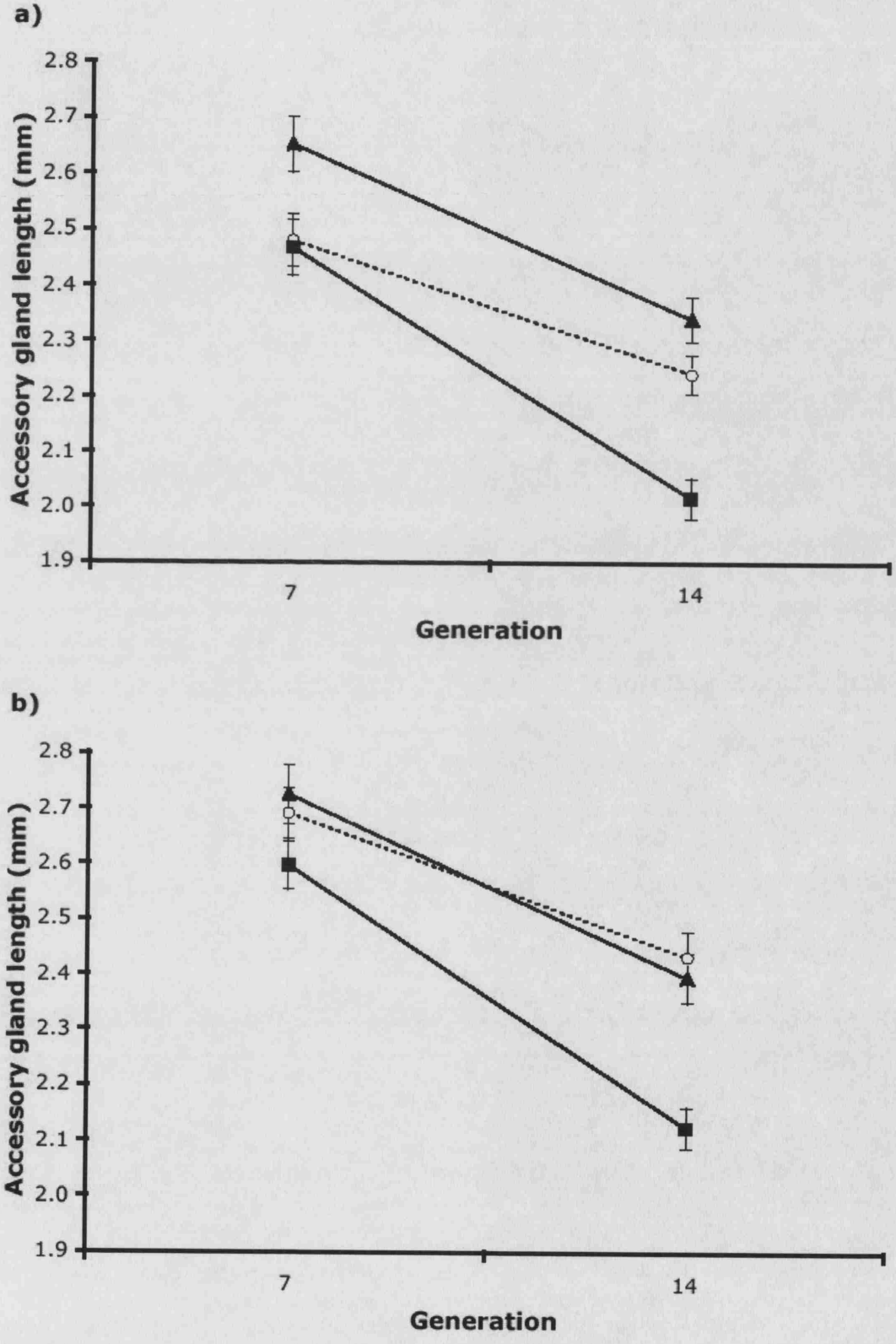
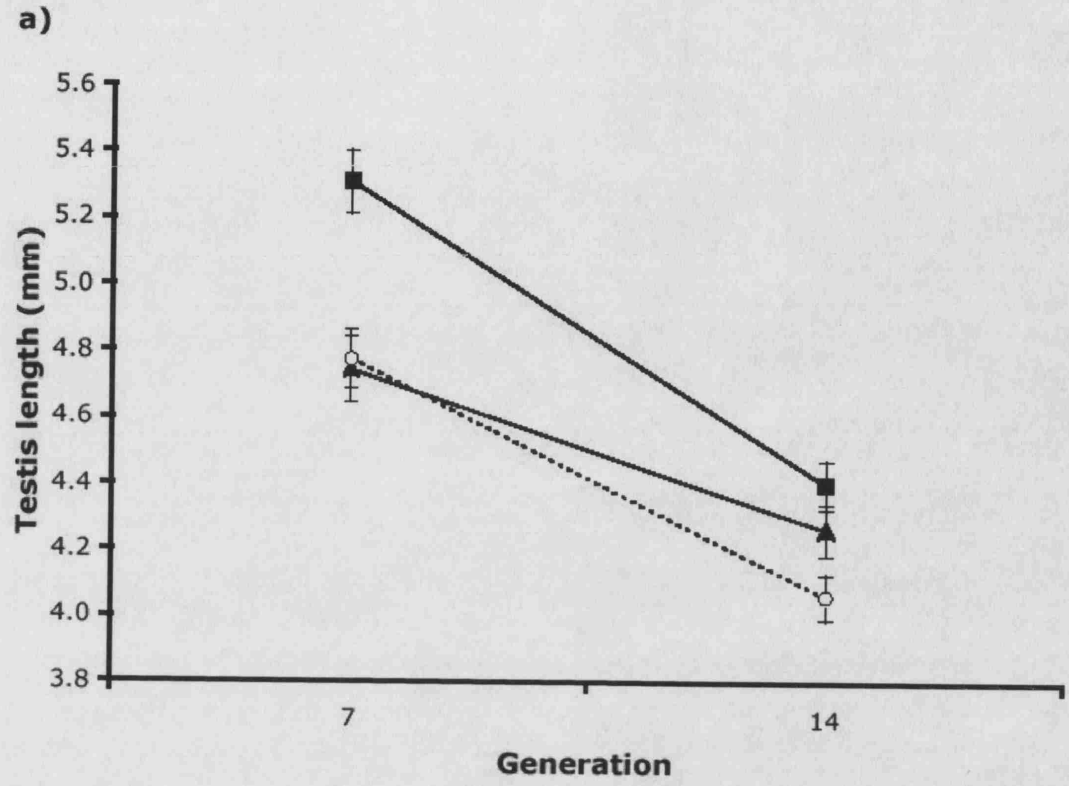

a)
Accessory gland length (mm)
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
7
14
Generation
b)
Accessory gland length (mm)
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
7
14
Generation

Figure 4.3


a)
Testis length (mm)
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
7
14
Generation

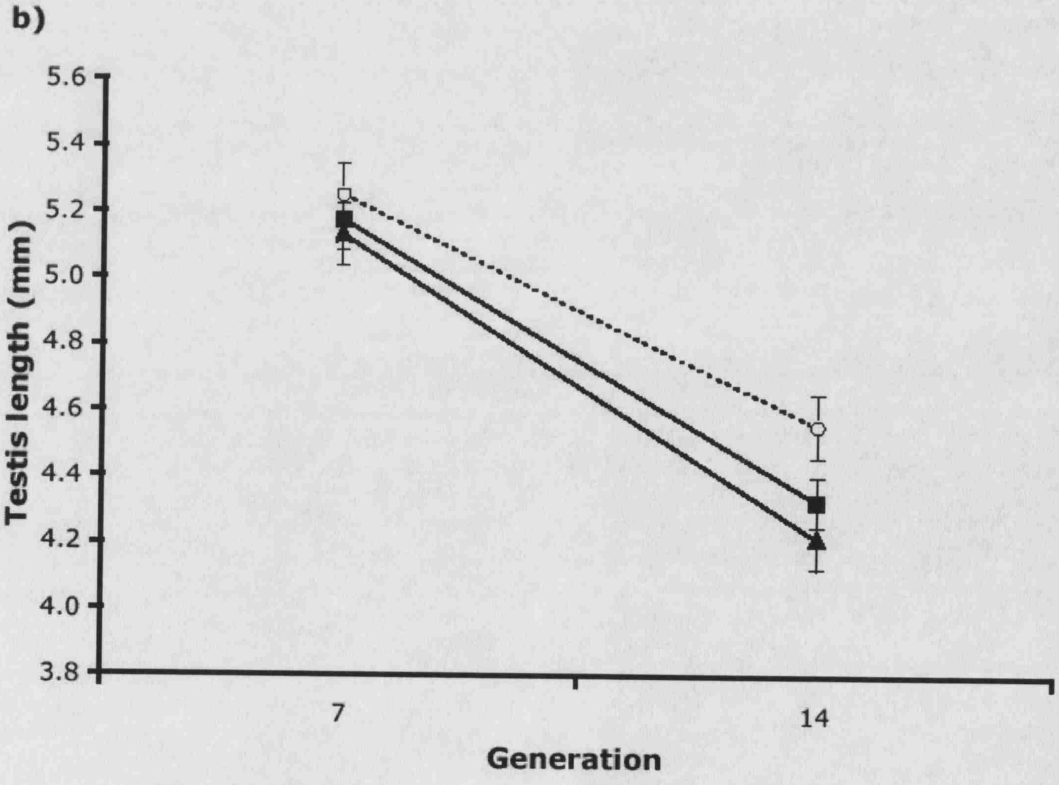
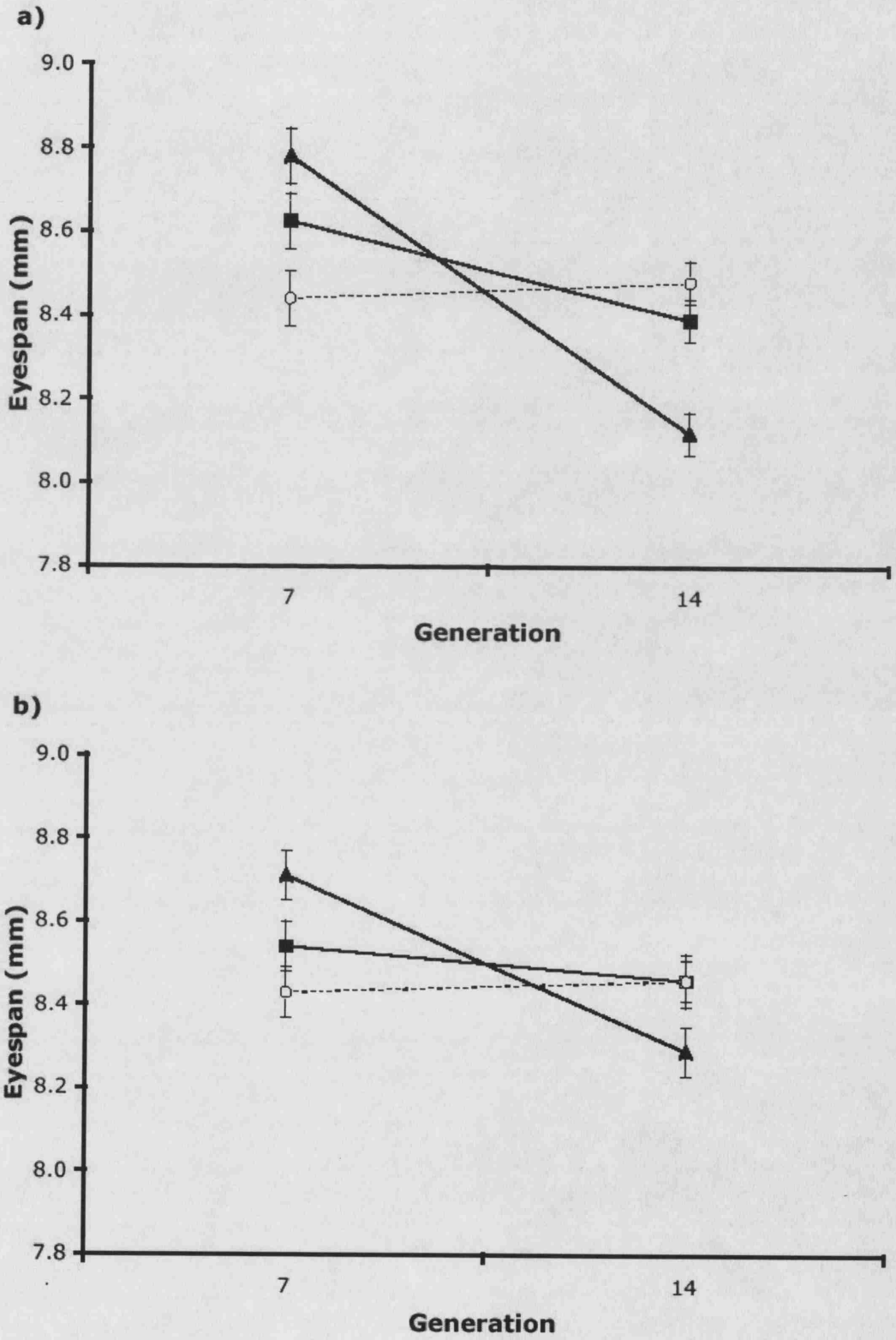

b)
Testis length (mm)
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
7
14
Generation

Figure 4.4


a)
9.0
8.8
8.6
8.4
8.2
8.0
7.8
Eyespan (mm)
7
14
Generation
b)
9.0
8.8
8.6
8.4
8.2
8.0
7.8
Eyespan (mm)
7
14
Generation

5

Mating-induced reduction in accessory reproductive organ size in the stalk-eyed fly *Cyrtodiopsis dalmanni*

5.1 Abstract

Internal reproductive organ size is an important determinant of male reproductive success. While the response of testis length to variation in the intensity of sperm competition is well documented across many taxa, few studies address the importance of testis size in determining other components of male reproductive success (such as mating frequency) or the significance of size variation in accessory reproductive organs. Accessory gland length, but not testis length, is both phenotypically and genetically correlated with male mating frequency in the stalk-eyed fly *Cyrtodiopsis dalmanni*. Here I directly manipulate male mating status to investigate the effect of copulation on the size of both the testes and the accessory glands of *C. dalmanni*. Accessory gland length was positively correlated with male mating frequency. Copulation induced a significant decrease in accessory gland size. The size of the accessory glands then recovered slowly over the next 8-48 hours. Neither testis length nor testis area was altered by copulation. These results reveal that the time course of accessory gland recovery corresponds to field observations of mating behaviour and suggest that accessory gland size may limit male mating frequency in *C. dalmanni*.

5.2 Introduction

There is a considerable body of evidence that reproductive organ size contributes to male reproductive success. This mainly derives from interspecific comparisons that have found positive relationships between testis size and the risk of sperm competition (Harcourt *et al.* 1981, Møller 1991, Gage 1994, Hosken 1997, Stockley *et al.* 1997). In addition, the direct manipulation of sperm competition intensity under experimental evolution has been shown to cause correlated changes in testis size in two species of Diptera (Pitnick *et al.* 2001, Hosken & Ward 2001). However, few studies have addressed the importance of internal reproductive organ size to other components of male reproductive success, or the significance of size variation in accessory reproductive organs which are often vital for sperm transfer, fertility, and essential for success in sperm competition (Leopold 1976, Gillot 2003).

In this chapter, I investigate how reproductive organ size may limit male mating frequency under conditions where males encounter high numbers of mating opportunities and are thus potentially at risk of sperm or seminal fluid depletion (Dewsbury 1982, Cartar 1985, Preston *et al.* 2001, Pitcher *et al.* 2005). Previous data support the hypothesis that male mating frequency can be limited by reproductive organ size in insects. For example, in dung flies, the length of the proximal section of the testis decreases with the number of copulations achieved in *Scathophaga stercoraria* (Ward & Simmons 1991) and increasing copula duration in *Sepsis cynipsea* (Martin & Hosken 2002). Testis mass is also lower in mated than in unmated Dawson's burrowing bees *Amegilla dawsoni* (Simmons *et al.* 2000). In contrast, accessory gland size, but not testis size, is phenotypically correlated with male mating frequency in *Drosophila melanogaster* (Bangham *et al.* 2002) and accessory glands become completely depleted and reduced in volume after 4-5 matings, leading to decreased fertility even though motile sperm remain in the seminal vesicles (Lefevre & Jonsson

1963, Hihara 1981). The ability to replenish reserves of sperm and seminal fluid likely further constrains male mating frequency (reviewed in Dewsbury 1982). Mating stimulates the replenishment of accessory gland products in *D. melanogaster* (Herndon *et al.* 1997). This resynthesis reaches a maximum after 2-4 hours and decreases to basal levels after 48 hours in *Drosophila funebris* (Baumann 1974).

In this study, I used the stalk-eyed fly *Cyrtodiopsis dalmanni* to test whether testis and accessory gland size are affected by mating. This is an ideal species, as males and females regularly mate at extremely high frequency (Baker *et al.* 2001, Baker *et al.* 2003, Chapter 4). Over 90% of matings occur in nocturnal aggregations which usually consist of a single male and a harem of several females (Wilkinson & Reillo 1994; up to 24 in the closely related species *Cyrtodiopsis whitei*, Lorch *et al.* 1993). Females join aggregations each evening and mate in the period immediately following dawn before dispersing (Burkhardt & de la Motte 1987, Lorch *et al.* 1993). During copulation, males transfer a single small spermatophore composed of sperm from the testes enveloped in accessory gland secretions (Kotrba 1996). Previous work has shown that accessory gland length, but not testis length, is phenotypically correlated with male mating frequency (Baker *et al.* 2003). Additionally, bidirectional artificial selection on male mating frequency resulted in a correlated response in accessory gland length but not in testis length (Chapter 4). While correlative evidence, whether phenotypic or genetic, indicates an association between accessory gland size and male mating frequency, it does not establish a direct physiological relationship between these two variables. In the current study, I provide direct evidence that mating induces a decrease in accessory gland, but not testis, size. Furthermore, I demonstrate that the timecourse of post-copulatory recovery of accessory gland size closely mirrors field observations of mating patterns in *C. dalmanni*.

5.3 Methods

5.3.1 General methods

The base stock was an outbred laboratory population of the stalk-eyed fly, *C. dalmanni*, collected from Gombak, Malaysia in 1993. The stock was maintained in large cages at high population size (typically more than 200 individuals per cage) and with a 1:1 sex ratio. Flies were fed ground corn medium and kept at 25 °C on a 12h/12h light/dark regime. The regime included a 15-min "dawn" period in which the culture room was illuminated by a single 60-W bulb. All observations of behaviour commenced at the start of this dawn period.

5.3.2 Manipulation of male mating status

Experimental flies were raised from eggs collected in groups of 13 from the population cages and allowed to hatch on moist cotton pads in Petri dishes containing at least 2g of ground corn (maize). Upon eclosion, flies were segregated according to sex and raised to sexual maturity in groups of 10 housed in 1.5L plastic pots on an *ad libitum* diet of ground corn. Mating observations were conducted using virgin males aged 6 weeks post-eclosion and virgin females aged 6-8 weeks post-eclosion. Males were randomly assigned to 5 mating status groups: unmated controls (n = 36), 0 hours recovery (n = 38), 2 hours recovery (n = 29), 8 hours recovery (n = 30), 24 hours recovery (n = 30), and 48 hours recovery (n = 30). At artificial dawn, individual males were added to 1.5L plastic pots containing 6 females, except for control males which were placed in empty 1.5L pots. The number of copulations over 40 seconds in duration occurring during the subsequent 60-minute period was recorded. Males that failed to mate during this observation period were discarded. Unmated control males and 0 hour recovery males were immediately placed on ice and dissected. Males

assigned to other recovery periods were moved individually to 500 ml plastic pots lined with a moist cotton pad and provided with ground corn until the appropriate time of dissection.

5.3.3 Morphological measurements

Males were dissected in a small amount of phosphate buffered saline on a microscope slide. Images of the accessory glands and uncoiled testes were captured using a monocular microscope connected via a video camera to a Macintosh computer with NIH Image (version 1.55). Length was measured by tracing a midline that longitudinally bisected each organ and the mean length of the two accessory glands or testes was used in analyses. Area was measured by tracing the outline of each organ and calculating the longitudinal surface area. Areas of both accessory glands were calculated and the mean used in analyses, but a single randomly chosen testis was measured per individual. Eyespan, was defined as the distance between the outer tips of the eyes.

5.3.4 Statistical analyses

Unless otherwise indicated, general linear models were used to analyse the determinants of reproductive organ size. Initial models included an intercept, male eyespan, recovery time and the eyespan × recovery time interaction. Recovery time was coded into models as an ordinal categorical variable. Stepwise elimination was used to remove terms that failed to significantly improve the fit of the model. Secondary analyses extended the models to include the number of copulations observed which required the exclusion of control males that did not copulate. Data sets did not deviate significantly from the assumptions of general linear modelling.

5.4 Results

I manipulated male mating status by providing males with the opportunity to mate with 6 virgin females for 60 minutes immediately following artificial dawn. Mated males were dissected at fixed times following this mating period (0 hours, 2 hours, 8 hours, 24 hours and 48 hours) and the sizes of their testes and accessory glands were compared to unmated control males. Mating resulted in a significant decrease in accessory gland length, but glands returned to their original size over the course of the next 8 to 48 hours. At average levels of male eyespan, included as a measure of body size to control for allometric variation ($F_{1,185} = 5.25, p = 0.0231$), mating status affected accessory gland length ($F_{5,185} = 4.72, p = 0.0004$). Post-hoc Tukey HSD tests revealed that males dissected immediately after mating or 2 hours after mating exhibited significantly smaller accessory glands than unmated controls. Gland length began to recover after 8 hours and by 48 hours after mating the accessory glands were significantly longer than immediately following mating (Fig. 1). Removing unmated control males from the analysis revealed a positive effect of mating frequency on accessory gland length ($b \pm$ s.e. $= 0.0228 \pm 0.0086$, $t_{149} = 2.67, p = 0.0085$) after controlling for the significant effect of recovery time ($F_{4,149} = 3.38, p = 0.0111$). Males mated a mean ± s.e. of 3.79 ± 0.20 (range: 1-12) times during the course of the 60 minute observation period, and mating frequency did not vary between groups dissected at different times ($F_{4,150} = 1.08, p = 0.3667$). Identical results were obtained when accessory gland length was replaced with area, but are not included as accessory gland length and the square root of area were highly positively correlated ($r_{90} = 0.926$, $p < 0.0001$).

Mating did not result in a decrease in testis length compared to unmated controls (Tukey HSD, Fig. 2). However, significant differences in testis length were detected between males measured at different recovery times ($F_{5,185} = 3.10, p = 0.0102$).

Post-hoc Tukey HSD tests revealed that males allowed to recover for 48 hours exhibited shorter testes than males allowed to recover for 2 or 24 hours. Testis length scaled with male eyespan ($F_{1,185} = 1.71, p = 0.0054$). Removing unmated males from the analysis failed to reveal any association between testis length and mating frequency ($F_{1,147} = 0.68, p = 0.4100$) after controlling for recovery time ($F_{4,147} = 3.66, p = 0.0071$) and eyespan ($F_{1,147} = 4.33, p = 0.0392$). Testis length and the square root of area were positively correlated ($r_{61} = 0.691, p < 0.0001$). As testis length explained less than half of the variance in testis area ($r^2 = 0.477$), I also directly compared testis area in males immediately after mating to that in unmated controls and detected no difference (mean ± s.e.: mated = 0.801 ± 0.021, unmated = 0.792 ± 0.020, $t_{65} = 0.276, p = 0.7834$).

5.5 Discussion

Male accessory gland size in *C. dalmanni* decreased dramatically following copulation and slowly recovered over the next 8-48 hours. After removing the effect of recovery time, accessory gland length was positively correlated with male mating frequency. Neither testis length nor testis area appeared to be altered by copulation; no significant difference in testis length was observed between mated and unmated males in the 48 hours following copulation.

Both male and female stalk-eyed flies mate frequently. In the current study, each male mated an average of 3.79 times (up to a maximum of 12) during the 60-minute observation period. Only 23.9% (37 out of 155) of males mated at least 6 times and therefore 76.1% (118 out of 155) of males failed to mate with all 6 virgin females provided. As females housed with three males will mate an average of 5.51 times during the 60 minutes following artificial dawn (Reguera *et al.* 2004), it is clear that male mating frequency was limited by physiological ability rather than the availability of willing females.

In the field, copulations occur primarily at dawn (Lorch *et al.* 1993, Wilkinson & Reillo 1994). My observations of the recovery of the accessory glands match this behavioural pattern, as 24 hours after copulation (i.e. the subsequent dawn period), the accessory glands had recovered their original pre-mating size. I found that the accessory glands had partially recovered after 8 hours which is consistent with the lower frequency of mating observed at dusk (Lorch *et al.* 1993, Wilkinson & Reillo 1994), whereas little recovery was observed in the hours immediately following copulation when flies leave mating aggregations to forage.

The two most plausible physiological constraints on male mating frequency in *C. dalmanni* are the availability of accessory gland products and the availability of sperm, both of which are required to produce spermatophores (Kotrba 1996). Several lines of evidence indicate that accessory gland size is more likely to limit mating frequency than testis length. First, I have demonstrated a decrease in accessory gland size following copulation and the subsequent recovery closely mirrors mating behaviour in the field. No significant reduction in testis size was observed in mated males compared to unmated controls. Second, our study confirms the results of a previous experiment showing that accessory gland length, but not testis length, is phenotypically correlated with male mating frequency (Baker *et al.* 2003). Third, bidirectional artificial selection on male mating frequency produced a correlated response in accessory gland length but not testis length (Chapter 4). However, I cannot exclude the possibility that some other currently unknown factor is the primary constraint on male mating frequency in *C. dalmanni.*

The full importance of the accessory glands in stalk-eyed fly reproduction is poorly understood. Accessory gland products form the casing of the spermatophore and consequently are necessary for sperm transfer (Kotrba 1996). Furthermore, accessory gland products appear to be important in sperm competition as seminal fluid can

decrease the viability of sperm from particular rival males in the female spermathecae (Fry & Wilkinson 2004). However, in contrast to *D. melanogaster* (Wolfner 2002), accessory gland products do not appear to play a role in sperm displacement (Fry & Wilkinson 2004), the inhibition of female remating (Grant *et al.* 2002) or the manipulation of female fecundity (Reguera *et al.* 2004). Consequently, the advantage of large accessory glands is likely gained through both increased mating frequency (by allowing males to produce more spermatophores over a given time period) and, potentially, greater success under sperm competition.

When receptive females are not limiting, male mating frequency in *C. dalmanni* is likely constrained by accessory gland size. Copulation causes a significant reduction in accessory gland size and replenishment of the depleted accessory glands follows a time course that is consistent with the observed daily peak in male mating frequency at dawn. There was no reduction in testis size following mating and therefore testis size appears to be of less importance in determining male mating frequency in this species.

5.6 References

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5.7 Figure legends

Fig 5.1 Reduction and subsequent recovery of accessory gland length following mating. Mean accessory gland length decreased from 2.10 mm to 1.85 mm following mating and was restored to the original size within 8-48 hours. Controls (con) were unmated (virgin) males. Columns not marked with the same letter are significantly different (Tukey HSD). Values shown are least squares means ± s.e. at average values of male eyespan.

Fig 5.2 Response of testis length to mating. Males dissected 48 hours after mating exhibited smaller testes than males dissected at 2 hours and 24 hours post-mating. Controls (con) were unmated (virgin) males. Columns not marked with the same letter are significantly different (Tukey HSD). Values shown are least squares means ± s.e. at average values of male eyespan.

Figure 5.1

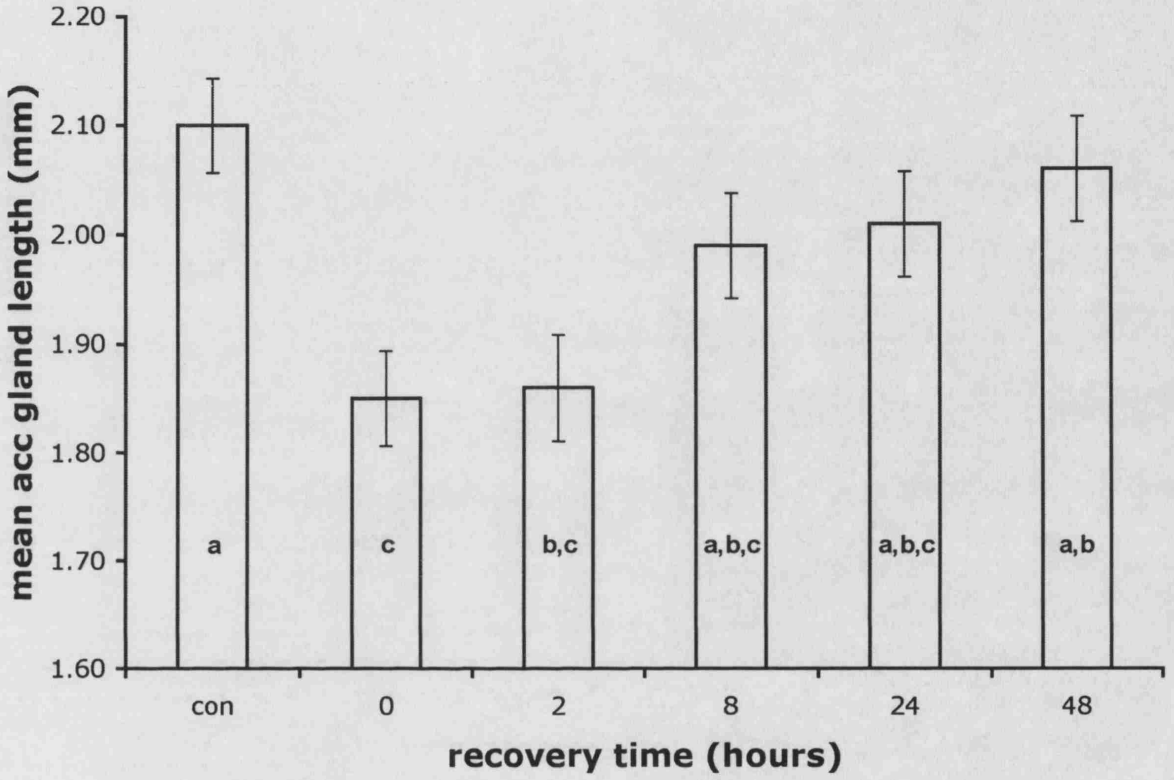
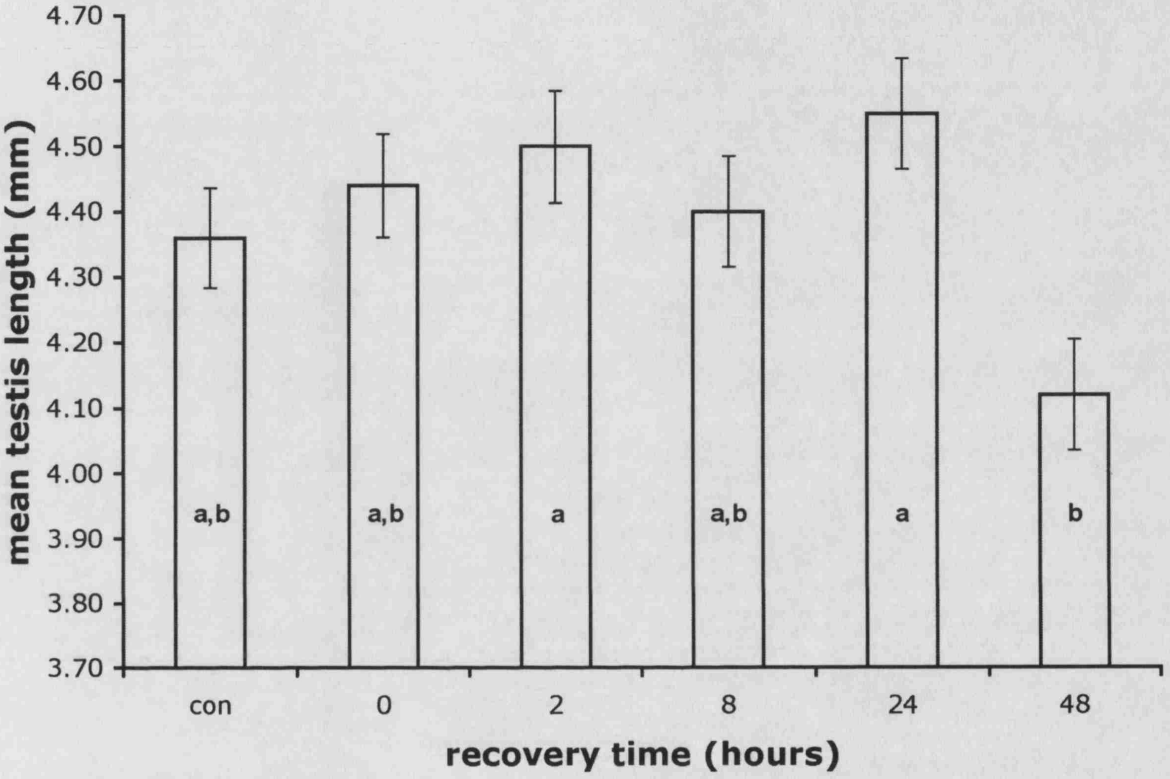

2.20
2.10
2.00
1.90
1.80
1.70
1.60
mean acc gland length (mm)
a
c
b,c
a,b,c
a,b,c
a,b
con
0
2
8
24
48
recovery time (hours)

Figure 5.2


mean testis length (mm)
4.70
4.60
4.50
4.40
4.30
4.20
4.10
4.00
3.90
3.80
3.70
a,b
a,b
a
a,b
a
b
con
0
2
8
24
48
recovery time (hours)

6

The effect of juvenile hormone on male and female reproduction in the stalk-eyed fly *Cyrtodiopsis dalmanni*

6.1 Abstract

Juvenile hormone (JH) is involved in an extremely broad range of developmental and physiological pathways across many insect species. Here I demonstrate that topical application of the JH analogue methoprene increases the strength of female preference for large eyespan males in the stalk-eyed fly *Cyrtodiopsis dalmanni*. Furthermore, topical methoprene application increases male accessory gland size and mating frequency relative to solvent-treated controls. As recent work indicates that JH might influence the expression of male eyespan in these flies, I review the evidence that JH can affect the development of insect structural traits in general and eyestalks in particular. The many roles played by JH in larval development and adult physiology suggest that JH might act as a physiological link between condition and condition-dependent sexual traits.

6.2 Introduction

The handicap hypothesis of sexual selection predicts that the intensity of a sexual signal will reflect the condition of the signaller. Variation in condition is thought to be determined by the genetic and environmental variance in a large number of underlying metric traits, in addition to environmental variance in condition itself (Price & Schluter 1991) and strong directional sexual selection for extreme values is predicted to increase the number of genes contributing to the signal (Pomiankowski & Møller 1995). However, how such a high number of genetic and environmental inputs can be physiologically translated into signal intensity remains unexplained.

The simplest mechanism would involve a physiological messenger whose activity is sensitive to genetic and environmental variation. In insects, a likely candidate is juvenile hormone (JH). JH is synthesized and released by paired neurosecretory glands called the corpora allata, which have become fused into a single structure known as the ring gland in higher Diptera (Richard *et al.* 1989). JH is involved in an extremely broad range of developmental and physiological pathways (Wheeler & Nijhout 2003) and is an important mediator of life history trade-offs (Dingle & Winchell 1997, Tatar & Yin 2001) in a large number of species. Most hormones, both lipid-soluble steroids (e.g. testosterone, ecdysone) and water-soluble peptides (e.g. insulin, eclosion hormone) have specific receptors to which they bind with high affinity. In contrast, JH belongs to an unusual class of lipid hormones called sesquiterpenoids (abscisic acid is another) that generally lack a high affinity receptor. Instead, JH exhibits low affinity interactions with a vast and diverse range of proteins including the nuclear receptors Methoprene-tolerant (Met, a possible transcription factor; Miura *et al.* 2005) ultraspiracle (USP, a component of the ecdysone receptor transcription factor; Fang *et al.* 2005), and the JHRE-binding protein (Zhou *et al.* 2002); membrane receptors involved in the protein kinase C (PKC) signal transduction

cascade (Yamamoto *et al.* 1988); and components of the mitochondrial electron transport chain (potentially resulting in the uncoupling of oxidative phosphorylation; Farkas & Sut'akova 2001). JH might even act directly on mRNA (Kushiro *et al.* 2003). The ability of JH to interact with such a wide range of signalling pathways and effector molecules likely underlies its diverse functions and importance to insect physiology and development (Wheeler & Nijhout 2003). Among adult insects, JH is a particularly important regulator of reproductive physiology. Here, I summarize the known effects of JH on female and male reproduction.

6.2.1 JH and female reproduction

Possibly the best characterized role of JH in female reproduction is the regulation of yolk protein (vitellogenin) biosynthesis and the uptake of vitellogenin by developing oocytes (Schal *et al.* 1997, Yin & Stoffolano 1997). In sexually mature virgin female *Drosophila melanogaster*, oogenesis is arrested at stage 9 of the 14-stage developmental cycle (Soller *et al.* 1999). This checkpoint occurs immediately prior to the uptake of vitellogenin by the maturing oocyte and consequently virgin females do not produce mature eggs. The increase in circulating levels of JH associated with mating (induced by the male accessory gland product sex-peptide; Moshitzky *et al.* 1996) stimulates the biosynthesis and uptake of vitellogenin thereby allowing oocyte development to progress past stage 9 to completion. Accordingly, topical application of JH can increase mature egg production in *D. melanogaster* (Salmon *et al.* 2001). Further support for the role of JH in fecundity comes from genetic studies. Different alleles at the aforementioned *Met* locus (which influences sensitivity to JH and its analogues; Shemshedini & Wilson, 1990) are associated with different levels of fecundity (Flatt & Kawecki 2004).

A small number of studies suggest that JH can influence female preference for male sexual signals. This has been investigated primarily by the surgical removal of the corpora allata (allatectomy) and the topical application of JH. Female phonotaxis in response to male calling song is reduced by allatectomy and restored by topical application of JH in the house cricket *Acheta domesticus* (Koudele *et al.* 1987). JH affects the stimulatory threshold of the L1 auditory interneuron (Stout *et al.* 1991) and the selectivity of the L3 auditory interneuron (Henley *et al.* 1992) to the male calling song in females. JH might also influence mate choice performed by males as injection of JH into allatectomized male noctuid moths (*A. ipsilon*) restores behavioural responsiveness and neuronal sensitivity to female sex pheromone (Anton & Gadenne 1999).

6.2.2 JH and male reproduction

Considerably less research has focussed on the importance of JH to male reproduction. Nevertheless, a clear role for JH in male accessory gland physiology has emerged. First, JH has been implicated in the post-eclosion development of the accessory glands (Bellés & Piulachs 1992) but this evidence is restricted to a small number of species (cf. Yin & Stoffolano 1997). Second, JH clearly promotes the synthesis of accessory gland proteins in a wide range of species (Gillot & Gaines 1992). This effect is best understood in *D. melanogaster*, where topical application of the JH analogue methoprene stimulates synthesis of accessory gland proteins in males (Herndon *et al.* 1997). Furthermore, JH deficiency or insensitivity is associated with low amounts of protein in the accessory glands. For example, null mutants at the *Met* locus exhibit 40-60% less total protein in their accessory glands than do wild-type controls (Wilson *et al.* 2003).

JH has been implicated in the regulation of male attractiveness in a number of species. Injection of JH induces pheromone production in allatectomized male cockroaches (*Nauphoeta cinerea*; Sreng *et al.* 1999) and increases the attractiveness of male pheromones in intact male mealworm beetles (*Tenebrio molitor*; Rantala *et al.* 2003). Topical application of JH increases pheromone production in virgin male Caribbean fruit flies (*Anastrepha suspense*; Teal *et al.* 2000). Moreover, JH-deficient *D. melanogaster* mutants – including *apterous* and *Met* – show greatly reduced courtship activity (Tompkins 1990, Wilson *et al.* 2003). The courtship activity of *Met* males can be partially restored through the topical application of methoprene. Finally, JH has been implicated in the development of structural secondary sexual traits; topical application of methoprene to late third instar male dung beetles (*Onthophagus taurus*) can induce horn expression in males destined to be hornless and increases horn length in horned males (Emlen & Nijhout 1999, 2001). Recent evidence also suggests a role for JH in the development of eyestalks in male stalk-eyed flies (C. Fry unpublished data, cited in Wilkinson *et al.* 2005; see Discussion).

6.2.3 JH and reproduction in stalk-eyed flies

Throughout this thesis, I have stressed the relationship between female preference, female fecundity, male internal reproductive organ size, mating frequency, and male eyespan in the stalk-eyed fly *Cyrtodiopsis dalmanni*. In this chapter, I describe the results of experiments on the effect of JH on the first four traits (through the topical application of methoprene to adult flies) and discuss the potential role of JH in reflecting condition both physiologically and through the development of male eyespan.

6.3 Methods

6.3.1 Flies and rearing

Flies were descended from adults collected in the field by A. Pomiankowski in 1993 and maintained in large numbers in population cages at 25°C on a 12L:12D photoperiod. Groups of 13 eggs were collected directly from population cages and placed in Petri dishes lined with moist cotton pads containing 5g of ground sweetcorn. After 15 days, pupae were collected and allowed to eclose. Adult flies were segregated by sex on the day after eclosion.

6.3.2 Effect of methoprene on female preference and fecundity

Groups of 10 large females (mean eyespan ± s.d. = 5.96 ± 0.14) were placed in 1500mL pots provided with ground corn medium *ad libitum*. Small females were discarded as large females exhibit stronger preference (Hingle *et al.* 2001a). Females were ice anaesthetised and treated with a single 1μl topical application to the ventral abdomen of either acetone alone or a 5μM solution of methoprene dissolved in acetone. Methoprene was applied on days 28, 35, and 42 after eclosion. To generate males with highly variable eyespan, larvae were reared under two levels of food stress (groups of 13 eggs were provided with either 5g or 0.39g ground corn; Cotton *et al.* 2004). Male eyespan was measured 2 days after eclosion and males were divided into two categories: large males (eyespan > 8.9 mm, mean ± s.d. = 9.12 ± 0.18 mm) and small males (eyespan < 7.5 mm, mean ± s.d. = 6.80 ± 0.52 mm). Intermediate males were discarded to maximise the probability of detecting variance in female preference (Hingle *et al.* 2001a). Males were maintained individually in 500mL pots and provided with ground corn *ad libitum* until the age of 6 weeks after eclosion.

Copulation preference was measured using males and females aged 6 weeks post-eclosion. Individual females were placed in 500ml pots 24 hours prior to the start of observations to acclimatize. Mating observations commenced at artificial dawn, when a single large or small male was added to each pot. The number of copulations occurring over 90 minutes was recorded at which point males were removed. The number of times each female copulated was measured on 4 consecutive mornings, alternating the size of the male presented daily. One half of the females were presented with a large eyespan male first, and the other half with a small eyespan male first. Copulation preference was calculated as the difference in the number of copulations with the 2 large males and the 2 small males, divided by the total number of copulations with all 4 males (after Hingle *et al.* 2001a,b). Females were frozen following their final mating period and later dissected to determine the number of mature eggs (defined as stages 12-14 using King's standard stages of oogenesis, King 1970) contained in their ovaries.

6.3.3 Effect of methoprene on male reproductive organ size and mating frequency

At eclosion, groups of 10 adult males were placed in 1500mL plastic pots and provided with *ad libitum* amounts of a low protein food consisting of a 25%:75% mixture (by mass) of ground corn and sugar (25% solution containing 3% carboxy-methylcellulose, and indigestible starch added to render the viscosity of the sugar solution similar to that of the ground corn). This stressful diet partially inhibits the growth of the testes and accessory glands (Chapter 2). Three days after eclosion, 1µl of an acetone-based methoprene solution was topically applied to the ventral surface of the abdomen. To test the effect of methoprene on male reproductive organ size, five different methoprene concentrations were used: a solvent control (acetone), 0.05µM, 0.5µM, 5µM, and 50µM methoprene dissolved in acetone. Approximately 40 males

were treated with each solution and dissected 28 days after eclosion. Accessory glands and the uncoiled testes were dissected out in phosphate buffered saline on a glass slide and the length of the line that bisected the middle of each organ was recorded using a videomicroscope attached to a computer equipped with NIH Image software. Eyespan was defined as the distance between the outer tips of the eyes.

In a separate experiment designed to test the effect of methoprene application on male mating frequency, males were treated with a single 1μl application of either acetone alone or 50μM methoprene dissolved in acetone. The application was performed 3 days after eclosion and males were reared as above for 28 days. To observe matings, individual males were housed with five virgin females in 1500 ml containers that had a central roosting string. Males and females were placed in their containers 24 hours prior to the first observation day to acclimatize. Mating frequency was scored as the total number of matings with a duration of more than 40 seconds (the minimum duration required for sperm transfer; Wilkinson & Reillo 1994) achieved in two 90 minute assay periods commencing at artificial dawn on consecutive days.

6.3.4 Statistical analysis

Female copulation preference and the number of eggs contained in the ovaries of females used in this experiment were analyzed using general linear models. Initial models included female methoprene treatment, eyespan, male order of presentation, and all possible interactions. Interaction terms were dropped if not significant through stepwise elimination but all main effects were included in final models. Male accessory gland length and testis length were also analyzed using general linear models. Initial models included methoprene treatment, eyespan and their interaction as predictors. The interaction term was dropped if not significant but all main-effects were included in final models. The distribution of male mating frequency could not be normalized and

was therefore subjected to non-parametric analysis. All statistical analyses were conducted using JMP statistical software package.

6.4 Results

6.4.1 Effect of methoprene on female preference and fecundity

Both methoprene-treated ($t_{61} = 4.87, p < 0.0001$) and acetone-treated females ($t_{61} = 2.36, p = 0.0217$) preferred large eyespan males over small eyespan males. However, females treated with methoprene exhibited stronger preference for large eyespan males than did females treated with acetone alone ($F_{1,120} = 5.31, p = 0.0229$, Fig. 6.1). Methoprene application did not affect the total number of copulations ($F_{1,120} = 1.41, p = 0.2375$). Female eyespan, included as a covariate, did not influence preference ($F_{1,120} = 0.91, p = 0.3430$) or copulation number ($F_{1,120} = 0.11, p = 0.7382$). The order in which males were presented to females (large eyespan male first or small eyespan male first) had a significant effect on preference ($F_{1,120} = 14.62, p = 0.0002$). Controlling for methoprene treatment, females presented with a small male first demonstrated weaker preference than females presented with a large male first (mean ± s.e.: small male first = 0.044 ± 0.035, large male first= 0.229 ± 0.035). Order did not influence the total number of matings ($F_{1,120} = 1.16, p = 0.2839$). Methoprene application did not affect the number of mature eggs contained in the ovaries of females used to study preference ($t_{117} = 0.27, p = 0.7877$).

6.4.2 Effect of methoprene on male reproductive organ size and mating frequency

Topical application of methoprene resulted in larger accessory glands ($F_{4,187} = 12.53, p < 0.0001$), but not testes ($F_{4,186} = 0.43, p = 0.7865$) relative to controls treated with acetone alone. Post-hoc Tukey HSD tests revealed that males treated with high

concentrations of methoprene (5μM and 50μM solutions) produced significantly larger accessory glands than males treated with acetone alone (Fig 6.2). No significant difference was detected between males treated with 0.05μM, 0.5μM or acetone alone. Eyespan was included as a covariate and was found to be positively associated with accessory gland length ($F_{1,187} = 6.24, p = 0.0133$) but not testis length ($F_{1,186} = 2.56, p = 0.1114$).

In a separate experiment, male mating frequency was measured over two 90-minute observation periods as the number of successful copulations achieved by individual males housed with 5 females. Males treated with methoprene mated at higher frequency than did males treated with acetone alone (Kruskal-Wallis test, $n_{met} = 18$, $n_{con} = 26$, $\chi^2 = 5.87$, d.f. = 1, $p = 0.0154$).

6.5 Discussion

6.5.1 Effect of methoprene on female preference and fecundity

I observed that female *C. dalmanni* exhibit copulation preference for large eyespan males (as defined by Hingle *et al.* 2001a,b); both acetone-treated and methoprene-treated females copulated more frequently with large eyespan males than with small eyespan males. This finding confirms the results of previous studies in both *C. dalmanni* and *C. whitei* (reviewed in Chapter 1). More importantly, I demonstrated that females treated with methoprene exhibited stronger copulation preference than did females treated with acetone alone. This result suggests a role for JH in determining the strength of female preference in *C. dalmanni*.

JH might influence the strength of female preference by modulating the underlying neural mechanism (e.g. Stout *et al.* 1991, Henley *et al.* 1992, Anton & Gadenne 1999). Female copulation preference for large eyespan males is stronger in

large eyespan females than it is in small eyespan females (Hingle *et al.* 2001a) likely because eyespan determines a female's ability to accurately assess male eyespan (de la Motte & Burkhardt 1983). JH might affect the sensitivity of neurons in the female visual system to male stimuli thereby increasing a female's response to male eyespan. Such a mechanism could explain the previously reported association between the strength of female copulation preference and female nutritional status. The strength of preference is reduced in females provided with a sucrose only diet and restored when provided with ground corn (Hingle *et al.* 2001b). As circulating levels of JH are highly sensitive to adult nutrition (see section 6.5.3), the relationship between nutrition and preference observed by Hingle *et al.* (2001b) might be mediated by JH. The observed relationship between JH and the strength of female preference suggests that female preference, like male eyespan, might be a condition-dependent trait.

Female *C. dalmanni* do not actively reject mating attempts by unwanted males. It is therefore difficult to disentangle female preference for large eyespan males from the innate physiological ability of large eyespan males to copulate at higher frequency than small eyespan males (Grant 2003). Variation in mating bias associated with female phenotype (such as eyespan, diet, or JH titre) provides better evidence that the higher frequency of copulation with large eyespan males is a consequence of female preference. Under the assumption that males will always mate as frequently as physiologically possible regardless of female phenotype, these differences are most consistent with variance in the strength of female preference associated with female eyespan and diet. However, one cannot currently rule out the possibility that males choose to mate more frequently with females exhibiting particular phenotypes.

Despite evidence that JH affects egg production in a wide range of species, I failed to detect any relationship between methoprene treatment and fecundity in *C. dalmanni*. In *D. melanogaster*, the progress of oogenesis beyond the arrest that occurs

at stage 9 is induced by an increase in JH titre associated with mating (Soller *et al.* 1999). In *C. dalmanni*, mating has no effect on female egg production (Reguera *et al.* 2004). Therefore, it is possible that JH is not required for the progress of oogenesis in this species. Alternatively, the acetone-treated control females used in the current study may have had high enough levels of circulating JH for normal egg production. It would be informative to determine if methoprene treatment can restore high fecundity in nutritionally deprived females.

6.5.2 Effect of methoprene on male reproductive organ size and mating frequency

Methoprene application was sufficient to increase accessory gland length, but not testis length, compared to acetone-treated controls when measured 28 days after eclosion. Adult males used in this experiment were fed a low quality diet known to partially inhibit internal reproductive organ growth (Chapter 1). A single topical application of concentrated methoprene solution (50μM) three days after eclosion was sufficient to restore accessory gland length of treated males to the level observed in males fed pure corn (Chapter 1: ~1.8mm). In contrast the testes of treated males remained considerably smaller than those of males fed pure corn (Chapter 1: ~4.3mm). Thus, while the relationship between nutrition and accessory gland size and might be regulated by JH a different mechanism regulates the relationship between nutrition and testis size.

Methoprene-treated males also exhibited higher mating frequencies than did acetone-treated controls. These results are consistent with findings described previously (Chapter 4, 5): mating frequency is closely associated with accessory gland size but not testis size. Indeed, the higher mating frequency of methoprene-treated flies is probably attributable to larger stores of accessory gland products and/or faster replenishment of depleted gland products.

Throughout this thesis, I have argued that male eyespan indicates fertility benefits to females through the signalling of both internal reproductive size and male mating frequency. Here, I have provided evidence that JH has a large influence on both accessory gland size and male mating frequency. If JH also influences the development of the eyestalks then it would provide a direct physiological connection between the signalling trait (eyespan), and the benefit signalled (fertility). In *C. dalmanni*, eyespan is determined during the larval stage while reproductive organ growth occurs primarily during the first 4 weeks of the adult stage. Consequently, if JH links eyespan and reproductive quality, larval and adult JH titres must be associated. Although little research has been conducted on cross life stage effects, there is evidence that larval factors can affect adult physiology. For instance, larval crowding increases adult heat stress resistance and longevity in *D. melanogaster* (Sørensen & Loeschcke 2001). However, Tu & Tatar (2003) found no effect of larval diet quality on adult JH titre in the same species.

6.5.3 JH and condition

The handicap hypothesis of sexual selection requires that signalling imposes a cost on the signaller, and that low-condition signallers pay greater marginal costs than high-condition signallers. These arguments can be extended to female choice when the strength of preference is condition-dependent. JH provides a mechanism to ensure honesty, as high JH titres generate important physiological costs. High JH titres are associated with decreased longevity in *D. melanogaster* (Tatar *et al.* 2001a) and the monarch butterfly (*Danaus plexippus*; Herman & Tatar 2001), reduced starvation resistance in the burying beetle (*Nicrophorus orbicollis*; Trumbo & Robinson 2004), humoral immune suppression in adult mealworm beetles (*Tenebrio molitor*; Rolff & Siva-Jothy 2002) and larval cotton leafworm moths (*Spodoptera littoralis*; Khafagi &

Hegazi 2001). Moreover, topical application of methoprene reduces the survival of adult *C. dalmanni* in a dose-dependent manner (personal observation). It is likely that these costs are considerably higher among individuals in low condition (those with low quality genotypes or raised in high stress environments) than among individuals in high condition (those with high quality genotypes or raised in low stress environments).

The costs associated with high JH titres require that individuals in poor condition have low levels of circulating JH. Indeed, the direct manipulation of condition can influence JH titre in larvae and adults. The volume of the corpora allata and circulating levels of JH are sensitive to the amount and quality of larval food (Emlen & Nijhout 2001 and references therein). For instance, female mosquito larvae (*Aedes aegypti*) raised under high nutritional stress, emerge from the puparium with low energy reserves and low rates of JH synthesis compared with females raised under low nutritional stress and in starved compared to sugar-fed adults (Caroci *et al.* 2004). Adults fed high quality diets exhibit higher levels of circulating JH in mosquitoes (Caroci *et al.* 2004), cockroaches (*Diploptera punctata*; Woodhead & Stay 1989) black blowflies (*Phormia regina*; Yin & Stoffolano 1997) and *D. melanogaster* (Tu & Tatar 2003). Recent microarray studies in *D. melanogaster* (Terashima & Bownes 2005) have demonstrated that starvation suppresses the expression of genes involved in JH synthesis such as *Vacuolar H^+ ATPase 44kDa subunit* (*Vha44*), and enhances the expression of genes involved in JH catabolism such as *Juvenile hormone epoxide hydrolase 2* and *3* (*Jheh 2* and *Jheh3*). Nutritional control of JH levels is probably regulated by the insulin signalling pathway. Insulin signalling mutants exhibit very similar phenotypes to nutritionally-deprived individuals (e.g. Kramer *et al.* 2003) and the wild-type phenotype can be restored in insulin signalling mutants by methoprene application (Tatar *et al.* 2001b). Importantly, genes in the insulin signalling pathway are important regulators of organ size, through the control of both cell size and cell

number (Brogiolo *et al.* 2001). Consequently, the interaction between JH and the insulin signalling pathway might control the development of structural ornaments in insects, including the eyestalks of *C. dalmanni.*

6.5.4 JH and eyestalk development

High circulating levels of JH provide reproductive benefits to males (larger accessory glands, higher maximum mating frequency). Females might gain directly from preferring to mate with males with high JH titres, but would require an external indicator of circulating JH levels to base this choice upon. In this section, I describe the influence of JH on the development of insect structural traits in general, and outline the evidence that JH might affect the development of eyestalks in stalk-eyed flies.

The eyestalks of adult stalk-eyed flies arise from the proliferation of cells in the eye-antennal imaginal disc, a group of cells in the larval body cavity derived from a simple invagination of the embryonic ectoderm (Hurley *et al.* 2001, Hurley *et al.* 2002). In many holometabolous insects, the duration of imaginal disc cell proliferation – and therefore the final size of the resulting adult structures - is regulated by circulating levels of JH and another important developmental hormone, ecdysone (reviewed in Emlen & Allen 2004). Typically, the proliferation of imaginal disc cells is delayed until the end of the larval period by high levels of circulating JH (and low levels of ecdysone). When larvae cease feeding and purge food from their guts in preparation for pupariation, circulating JH titres decrease rapidly triggering the onset of imaginal disc cell proliferation. Proliferation continues until the cells are exposed to high levels of ecdysone (in the absence of JH) during the prepupal period. A subsequent transient increase in JH titre initiates a second period of proliferation which continues until JH levels fall at the onset of the pupal period, proliferation stops and the imaginal disc cells begin to differentiate. To summarize: JH inhibits proliferation when

ecdysone levels are low during the laval period, but stimulates proliferation when ecdysone levels are high during the prepupal period.

Different imaginal discs, and even different regions of a single imaginal disc, exhibit different sensitivities to JH (Truman & Riddiford 2002). For instance, the wing discs of Lepidoptera are largely insensitive to juvenile hormone allowing proliferation to begin early in the larval period, when JH levels are high (Truman & Riddiford 2002). This extended period of proliferation is required for the production of large adult structures such as butterfly wings. Low sensitivity of imaginal disc cells to JH has evolved independently at least six times in the insects (Truman & Riddiford 1999).

In the dung beetle *O. taurus*, large males exhibit different sensitivity to JH than do small males and females. Dung beetle horns develop from the outbudding of a selected region of the larval epidermis termed the horn primordium (similar to an imaginal disc; Moczek & Nagy 2005). Individuals that fail to reach a certain threshold bodysize by a specific time in larval development experience a pulse of ecdysone that greatly reduces the sensitivity of the horn primordium to JH (Emlen & Nijhout 2001). Consequently, when circulating JH levels rise later in development (when ecdysone levels are high), the cells of the horn primordia of large males proliferate resulting in large horns while the cells of the horn primordia of small males and females do not proliferate resulting in small or absent horns. Topical application of methoprene during this second critical period (when ecdysone levels are high) extends the duration of horn primordium cell proliferation thereby increasing horn length in methoprene-treated males relative to control-treated males (Emlen & Nijhout 1999). However, the physiological mechanism regulating horn development is clearly more complicated than described as the lowest dose of topically applied methoprene that affects horn length also causes treated individuals to die as pupae (Emlen & Nijhout 1999, Moczek & Nijhout 2002).

Similar to the wing discs of Lepidoptera, the imaginal discs of *D. melanogaster* and other higher flies begin proliferating very early in the larval period (Truman & Riddiford 1999). The insensitivity of fly imaginal discs to JH is likely an adaptation allowing extremely rapid larval development. Adult structures not derived from imaginal discs, including the gut, musculature, central nervous system and abdominal epidermis remain sensitive to JH. Treatment of *D. melanogaster* prepupae with JH or a JH-analogue impairs the development of these structures and results in the death of pharate adults (Restifo & Wilson 1998). The developmental defects associated with JH-treatment are usually quite minor, and the death of pharate adults is thought to be primarily associated with JH-induced misexpression of genes involved in pupa-to-adult metamorphosis including *Broad Complex* and *Deformed* (Restifo & Wilson 1998). Intriguingly, *Deformed* is an excellent candidate for the development of hypercephaly in the Diptera (DeSalle & Carew 1992, Chapter 1).

The involvement of JH in the development of hypercephaly remains unknown. The death of individuals treated with JH or a JH-analogue as pharate adults presents a major obstacle to research in stalk-eyed flies. Dung beetles treated with methoprene die during the pupal period, but horn length can be measured because the horns are fully developed in pharate adults. In contrast, the eyestalks of Diopsid flies must be 'inflated' after emergence from the puparium (Buschbeck *et al.* 2001) and therefore cannot be measured in dead pharate adults. Consistent with results from dung beetles and *D. melanogaster*, my own attempts to manipulate eyespan through the topical application of methoprene to *C. dalmanni* have resulted in the death of treated individuals as pharate adults (personal observation). These individuals exhibited identical deformities to *D. melanogaster* treated with methoprene (i.e. methoprene syndrome, Restifo & Wilson 1998). Although late third instar larvae and prepupae can survive topical application of very low concentrations of methoprene, these treatments

have no effect on male eyespan (personal observation). However, as indicated above, other reports suggest that not only can stalk-eyed fly larvae survive treatment with methoprene but that topical application of methoprene to third instar larvae increases the eyespan of small-bodied males (C. Fry unpublished data, cited in Wilkinson *et al.* 2005).

A much better understanding of the role of JH in larval development and the proliferation and differentiation of the eye-antennal disc will be necessary to properly evaluate the influence of JH on eyespan in stalk-eyed flies. The death of pharate adults following exposure to the JH-analogue methoprene must be addressed in both stalk-eyed flies and dung beetles. Several possible mechanisms might explain how JH could influence proliferation of the eye-antennal disc, and specifically the section of the disc that gives rise to the eyestalks, without disrupting the development of other tissues. For instance, in the tobacco hornworm moth *Manduca sexta*, the corpora allata stops secreting JH and instead secretes juvenile hormone acid (JHA) into the haemolymph towards the end of the larval feeding period (Janzen *et al.* 1991). JHA is largely inactive in both *M. sexta* (Ismail *et al.* 1998) and *D. melanogaster* (JH III and JH III bisepoxide are the active juvenoids in higher Diptera; Richard *et al.* 1989). However, JHA can be converted into an active form in specific tissues through the expression of the enzyme juvenile hormone acid methyltransferase in the target tissues. Juvenile hormone acid methyltransferase is present in high levels in certain imaginal tissues in *M. sexta* (Sparagana *et al.* 1985) and an orthologue has been identified in *D. melanogaster* (Shinoda & Itoyama 2003). This mechanism could be tested in stalk-eyed flies by treating late third instar larvae with JHA or a JHA-mimic instead of methoprene which mimics the effects of JH.

6.5.5 Summary

The influence of JH on male and female reproduction described in this study, coupled with the potential role of JH in the development of the eyestalks, suggest that JH might act as a physiological "linchpin" for condition-dependent sexual selection in *C. dalmanni*. However, many further experiments are required to confirm this hypothesis. Future work should attempt to provide direct measures of JH titres in adult males with different eyespan, and demonstrate that high JH titres are more costly to small eyespan males than to large eyespan males.

6.6 References

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6.7 Figure Legends

Fig 6.1 Effect of topical application of the juvenile hormone analogue methoprene on female copulation preference for large or small eyespan males. Copulation preference greater than 0 indicates bias toward large eyespan males, less than 0 indicates bias toward small eyespan males, and equal to 0 indicates random mating. Values represent mean ± s.e.

Fig 6.2 Effect of topical application of the juvenile hormone analogue methoprene on (a) accessory gland length and (b) testis length in males 28 days after eclosion. All methoprene solutions were prepared in acetone, and the 0μM group represents solvent controls. Values represent least squares mean ± s.e.

Figure 6.1

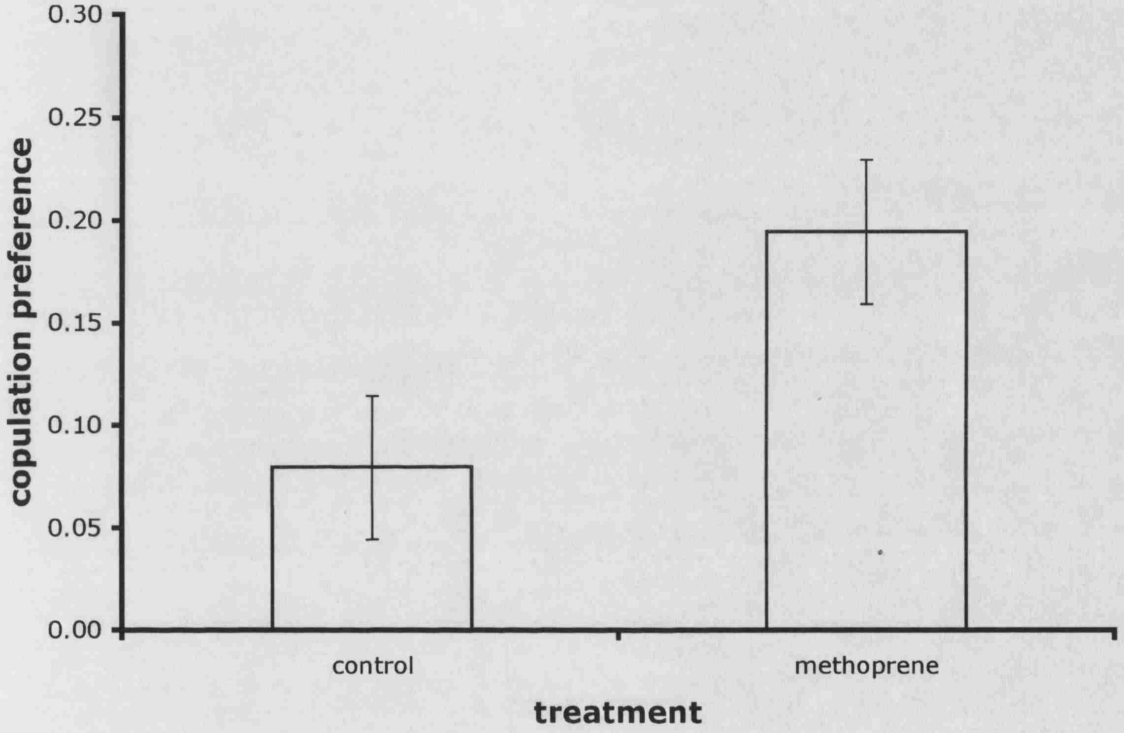
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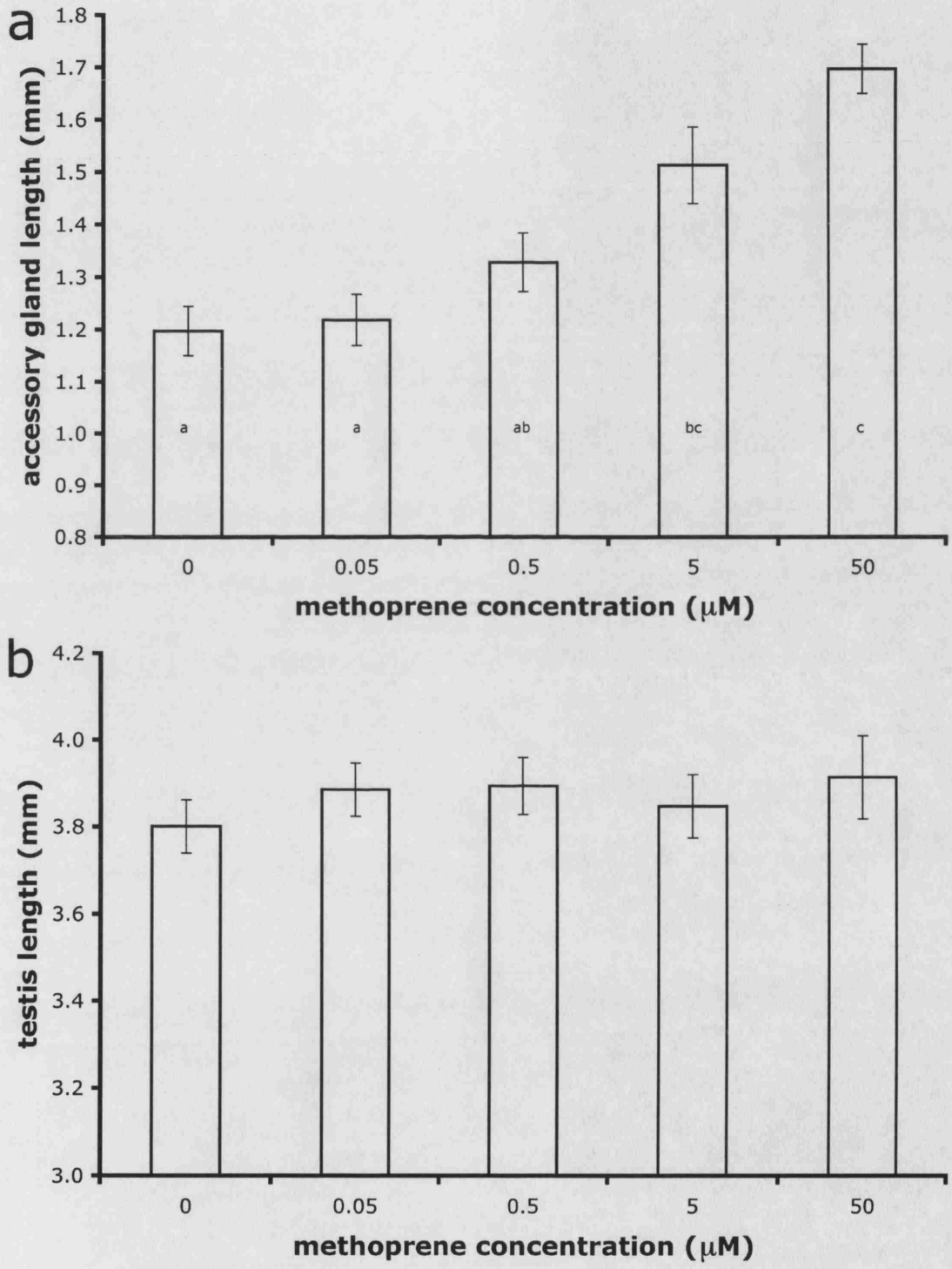
Figure 6.2


a
accessory gland length (mm)
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methoprene concentration (μM)
b
testis length (mm)
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4.0
3.8
3.6
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0
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methoprene concentration (μM)

7

General discussion

7.1 Overview

Recent research on stalk-eyed flies has focussed on testing the assumptions of indirect benefit models of female preference for exaggerated male sexual ornaments (cf. Wilkinson 2001, Cotton 2004). While this approach has produced many important results, adopting a narrow focus on a single interpretation risks overlooking other important alternative and/or complementary explanations. In this thesis, I have tested neglected hypotheses for the evolution of stalk-eyed fly reproductive behaviour. Here, I first provide a summary of my principal findings in a manner that illustrates how the results described in each chapter fit together. Second, I place these results in a broader view of sexual selection in stalk-eyed flies and consider complementary hypotheses (i.e. indirect benefits) for the evolution of female preference for large male eyespan. Finally, I propose several avenues for future research that stem from the ideas presented in this thesis.

7.2 Summary of principal findings

7.2.1 The phenotype-linked fertility hypothesis

Many studies have claimed that male sexual ornaments signal direct fertility benefits to females. These claims are typically based on a simple positive correlation, usually measured in a single environment, between the size of a male ornament and some measure of male reproductive quality. I demonstrated that the *C. dalmanni* mating system fulfils three criteria required for convincing tests of the phenotype-linked fertility hypothesis (Chapter 2). First, I confirmed the results of previous studies that females prefer highly ornamented males, in this case those with large eyespan (Chapter 6). Second, I showed that females housed with large eyespan males exhibited

higher fertility than females housed with small eyespan males. Third, I found a positive relationship between male eyespan and internal reproductive organ length not only in flies maintained under benign conditions but also in flies maintained under several other levels of nutritional stress. Together these results provide convincing evidence that male eyespan reliably signals reproductive quality and that preference for males with exaggerated eyespan can directly increase female reproductive success. The fertility benefits associated with choice of with large eyespan males are not gained on a per-mating basis (Chapter 3) indicating that the reproductive advantage of large eyespan males is achieved through their ability to copulate at higher frequencies than small eyespan males.

7.2.2 Determinants of male mating frequency

I have proposed that females can gain direct fertility benefits from choosing large eyespan males as mating partners because large eyespan males are capable of mating more frequently than small eyespan males. This hypothesis requires that male mating frequency is limited, at least in part, by male physiology rather than female receptivity alone. Alternatively, it is possible that variance in male mating frequency can be entirely attributed to variance in male attractiveness.

I tested the hypothesis that male mating frequency can be limited by male physiological ability using males artificially selected for increased and decreased mating frequency (Chapter 4). During artificial selection, variance in male eyespan was constrained to minimize the influence of female choice. Male mating frequency was assayed using a constant, non-limiting, but ecologically realistic number of females. There was a rapid bidirectional direct response of male mating frequency to artificial selection. This result reveals that males can be limited by their physiological capacity

to mate and that the mating frequency of most males in the laboratory population is below the physiological maximum.

In an attempt to identify the physiological constraints on male mating frequency, I looked for correlated responses to selection in the sizes of the male internal reproductive organs (Chapter 4). While no difference was observed between the selected lines in testis length, the accessory glands of males selected for high mating frequency were larger than those of males selected for low mating frequency. Thus, male mating frequency is genetically correlated with male accessory gland size. I further demonstrated that accessory gland size, but not testis size, decreases following mating (Chapter 5). The timecourse of the subsequent post-copulatory recovery of accessory gland size closely mirrors the temporal distribution of matings in the field. These results indicate that the positive relationship between male eyespan and accessory gland size (Chapter 2) enables large eyespan males to copulate at a higher maximum physiological frequency than small eyespan males.

7.2.3 Female fertility and male strategic allocation of ejaculates

When offspring production is partly limited by the ability of males to produce ejaculates, males are expected to distribute their reproductive effort in a manner that maximises their opportunities to gain fertilisations. I used three lines of evidence to test this hypothesis in *C. dalmanni* (Chapter 3). First, in order to determine the potential number of fertilisations offered by a mate, males require an external phenotypic indicator of female reproductive quality. I showed that female egg production is positively associated with female eyespan. Second, I demonstrated that large eyespan females laid a significantly higher number of fertile eggs following a single copulation than did small eyespan females suggesting that males increase their reproductive effort when mating with large eyespan females. Third, I re-analyzed existing data on the sizes

of spermatophores that males allocated to large and small eyespan females. Under certain circumstances, males transferred larger spermatophores to large eyespan females than they did to small eyespan females. Thus, males are able to tailor their ejaculates in an adaptive manner, expending more reproductive effort when mating with females offering a greater number of fertilisation opportunities.

7.2.4 The physiological basis of condition-dependent signalling

How the many different genetic and environmental inputs that determine condition are translated into the expression of a sexual ornament remains one of the most important questions in the field of sexual selection. I have proposed that juvenile hormone (JH) could act as a physiological link between reproductive quality and eyespan in male *C. dalmanni* (Chapter 6). Topical application of methoprene was used to determine the effects of JH on male reproductive quality in this species. I found that methoprene application increases accessory gland size and male mating frequency relative to solvent-treated controls. Although my attempts to manipulate male eyespan through the topical application of methoprene were not successful, the idea that JH influences eyespan in *C. dalmanni* remains viable. Intriguingly, the topical application of methoprene also increased the strength of female copulation preference for large eyespan males relative to solvent treated controls. These results suggest that similar physiological mechanisms might regulate condition-dependent signalling in males and preference for these signals in females.

7.3 Male and female effects on mating frequency

As female *C. dalmanni* lack any overt rejection response, it is difficult to separate the effects of males and females on mating frequency. Grant (2003)

demonstrated that large eyespan (>8.6mm) males mated roughly twice as frequently as did small eyespan (<6.4mm) males, but this difference could be caused by either a higher physiological capacity for multiple mating in large eyespan males, or the increased receptivity of females paired with large eyespan males. While female preference for large eyespan males surely plays a role, there is ample evidence that male mating frequency is physiologically constrained. First, when variation in male eyespan is experimentally controlled, male mating frequency is strongly influenced by male accessory gland size (Chapters 4-6). Since internal reproductive organ size cannot be directly assessed by females, these results strongly suggest that accessory gland size physiologically constrains maximum male mating frequency. Second, all assays of male mating frequency were conducted using virgin females and high female:male sex ratios. When the cost of mating to females is low, as it is in *C. dalmanni* (Reguera *et al.* 2004), ejaculate-limited females are not expected to refuse copulations with low quality males (Halliday 1983, Gabor & Halliday 1997, Pitcher *et al.* 2003); laying eggs fertilized by small eyespan males is obviously preferable to laying unfertilized eggs.

The limited ability of males to produce ejaculates and the corresponding high female mating frequencies do not reduce the importance of female choice in *C. dalmanni*. Indeed, the high cost of laying unfertilized eggs should impose strong selection on females to choose partners capable of providing an ejaculate. In the field, males usually arrive at nocturnal aggregation sites before females (Burkhardt & de la Motte 1988), allowing females the opportunity to compare the eyespan of males at different aggregation sites. As large eyespan males are capable of mating at higher frequency than are small eyespan males, females should roost in higher numbers with large eyespan males to maximize their chances of obtaining a spermatophore. However, even large eyespan males can only mate a limited number of times. Consequently, females might benefit more from roosting with unpaired small eyespan

males than from roosting with large eyespan males with large harems. Unlike most lek-mating species, where mating success is often restricted to a small number of males (or even a single individual) in a subpopulation (Andersson 1994), male mating success in sexually dimorphic stalk-eyed flies is distributed between males in proportion to male eyespan. Although large eyespan males attract more females, small eyespan males do obtain mates. Observations of *C. whitei* aggregations in the field revealed that males with 10mm eyespans were usually accompanied by 4 females while males with 7mm eyespans usually paired with a single female (Burkhardt & de la Motte 1988).

7.4 Genetic benefits signalled by eyespan

Regardless of the relative strengths of selection for direct and indirect benefits, the existence of one type of benefit does not preclude the other if the correlations between the male ornament and each type of benefit are in the same direction. Apart from fertility assurance, females choosing to mate with large eyespan males almost certainly obtain heritable genetic benefits. In this section, I discuss the potential genetic benefits of female preference for large eyespan males.

7.4.1 The Fisher process

Females might benefit from mating with attractive males simply through the production of attractive sons. Wilkinson (1993) found that artificial selection for increased and decreased male eyespan in *C. dalmanni* produced a rapid bidirectional response suggesting a large additive genetic component to male eyespan. The selection intensity used by Wilkinson was roughly double that estimated in the field (calculated as the covariance between the average harem size and the ratio of male relative eyespan over its standard deviation; Wilkinson & Reillo 1994). Consequently, females that mate with males with large eyespan will produce sons with large eyespan that enjoy the same

reproductive advantage as their fathers. Moreover, male eyespan and female preference are genetically correlated as selection on male eyespan generated a correlated response in the direction of female preference (Wilkinson & Reillo 1994). If the correlation between male eyespan and female preference arises from linkage disequilbrium rather than pleiotropy, then Fisher's self-reinforcing process (see Chapter 1) might contribute to the evolution of extreme sexual dimorphism in eyespan. The nature of the genetic correlation between male eyespan and female preference requires further investigation.

7.4.2 Good genes

I have only considered the direct benefits obtained from males capable of mating at high frequency. In Chapter 4, I reported high levels of additive genetic variance in male mating frequency, and male mating frequency is phenotypically correlated with male eyespan (Grant 2003). If male mating frequency exhibits positive genetic covariance with male eyespan, then females mating with large eyespan males would produce sons capable of mating at high frequency. Similarly, the ability of males to produce large eyespan under high larval stress exhibits high levels of genetic variance (David *et al.* 2000, Cotton 2004) suggesting that eyespan might indicate genetic differences in larval stress resistance. Consequently, females might benefit from mating with large eyespan males through the production of stress resistant sons. Indeed, if most genes in the genome make a small contribution to the determination of male eyespan, as is expected under genetic models of condition-dependent sexual selection, females will benefit from mating with large eyespan males by producing sons, and likely daughters, of high overall genetic quality.

7.4.3 Suppression of X-linked meiotic drive

Wilkinson *et al.* (1998) has argued that X chromosome-linked meiotic drive

might underlie female preference for large eyespan males. If females avoid mating with SR males, then they will produce more sons who have enhanced reproductive success in a female-biased population (Lande & Wilkinson 1999). Artificial selection on male eyespan to body length ratios in *C. dalmanni* resulted in altered offspring sex ratios compared to males from stock populations (Wilkinson *et al.* 1998). The offspring of males from two replicate lines selected for large eyespan were less frequently female-biased, and more frequently male-biased, than those produced by stock population males. In contrast, the offspring from males from one of two replicate lines selected for small eyespan were more frequently female-biased than those produced by stock population males. The sex ratios of offspring of males from the second replicate line selected for small male eyespan did not differ from the stock population. Wilkinson *et al.* (1998) interpreted these changes as evidence of a genetic association between male eyespan and a Y-linked suppressor of meiotic drive allowing females to use eyespan as an indicator of male insensitivity to drive.

There is little evidence that male eyespan is an indicator of Y-linked insensitivity to drive. First, Wolfenbarger & Wilkinson (2001) failed to find any effect of the Y chromosome on male eyespan, suggesting that eyespan-determining loci are not linked to any suppressor of meiotic drive on the Y chromosome. Second, while some males are clearly insensitive to drive, there is no strong evidence that this insensitivity is caused by a Y-linked, as opposed to an autosomal, suppressor (Presgraves *et al.* 1997). Finally, because male resistance to meiotic drive must carry a cost, theoretical models have demonstrated that female preference for males with Y-linked suppressors (but not suppressors at other chromosomal locations) actually decreases female fitness (Lande & Wilkinson 1999, Reinhold *et al.* 1999). At equilbrium, a male with a resistant Y and a male with a sensitive Y must produce equal numbers of sons (otherwise, there would be continued selection). Resistant males will

produce equal numbers of sons and daughters, but sensitive males will produce more daughters than sons due to meiotic drive. Consequently, resistant males will produce fewer progeny overall at equilibrium than will sensitive males. Females choosing to mate with resistant males would therefore suffer reduced reproductive success. Clearly, alternative interpretations of the results of Wilkinson *et al.* (1998) are necessary.

Meiotic drive elements are usually located in chromosomal inversions; this protects against the decoupling of drive and insensitivity alleles ensuring that driving chromosomes cannot destroy themselves (Hurst & Werren 2001). When meiotic drive occurs at low frequency there will be little opportunity for recombination in this inverted chromosomal region resulting in a gradual increase in the number of recessive mutations linked to the drive allele. When X-linked drive increases in frequency, these recessive mutations will be expressed in females with two driving X chromosomes often resulting in sterility or inviability (Hurst & Werren 2001). The frequency of sterile or inviable females with two driving X chromosomes will increase dramatically under inbreeding.

In the artificial selection experiment described by Wilkinson *et al.* (1998) only 10 males from each replicate line contributed offspring to the next generation, resulting in high levels of inbreeding and therefore strong selection against drive. The decrease in the frequency of drive observed in the two replicate lines selected for large eyespan to body length ratios might be attributable to selection against homozygous females rather than any genetic association between male eyespan and a Y-linked suppressor. This could be easily determined, as unselected control lines would experience similar levels of inbreeding to selected lines and should therefore also exhibit a decrease in the frequency of drive. Unfortunately, Wilkinson *et al.* (1998) failed to report changes in the unselected control lines. As artificial selection had no effect on the frequency of drive in one of two replicate lines selected for small eyespan to body length ratios, the

actual evidence supporting an association between male eyespan and the frequency of meiotic drive stems from the increase in the proportion of female biased offspring in a single replicate line selected for small eyespan to body length ratios. While this result could be due to drift, it might also point to linkage between eyespan loci and the drive locus on the X chromosome. The X chromosome influences male eyespan (Wolfenbarger & Wilkinson 2001) and female choice for males with drive-free X chromosomes can be advantageous when resistance to meiotic drive is absent (Lande & Wilkinson 1999, Reinhold *et al.* 1999).

The results of Wilkinson *et al.* (1998) might best be explained through the condition dependence of male eyespan (Reinhold *et al.* 1999). Males with large eyespan (i.e. those in good condition) might pay lower marginal costs for drive resistance. Consequently, selection for increased eyespan would result in a higher frequency of males capable of surviving the viability costs of resistance, while selection for decreased eyespan would result in a lower frequency of resistant males. Under this scenario, there is no requirement that eyespan-determining genes are linked to the drive locus. Models that require close genetic linkage between male eyespan and drive are implausible because male eyespan is likely determined by a large number of genes widely distributed throughout the genome. Consequently, any genes closely linked to the drive element will only make a small contribution to male eyespan. Thus, female preference for drive-free males might actually be explained by traditional condition-dependent sexual selection.

7.5 Future directions

7.5.1 Characterisation of the components of the ejaculate

The ability of males to produce spermatophores likely limits male and female reproductive success in *C. dalmanni* (Chapters 4 and 5), suggesting that ejaculate production is metabolically or energetically expensive. However, the roles played by the accessory gland products in reproduction remains poorly understood. The female traits typically altered by male accessory gland products in insects (regulation of oogenesis and oviposition, refractoriness to mating, and viability; reviewed in Gillott 2003) are unaffected by mating in *C. dalmanni* (cf. Chapters 5 & 6). The only clear function of the accessory gland products in this species is the formation of the casing of the spermatophore which is required for successful sperm transfer (Kotrba 1996). Diopsids in the genus *Diopsis* have either partially formed spermatophores or lack them altogether (Kotrba 1996). If the sole function of the accessory glands in stalk-eyed flies is the production of the spermatophore casing then we would expect members of this genus to exhibit greatly reduced accessory glands. This could be investigated through simple anatomical comparisons.

Most recent research on insect accessory glands has focussed on peptides that benefit males by reducing the success of rivals or by manipulating the reproductive interests of females (e.g. Wolfner 2002). As a consequence, accessory gland products that modulate male fertility have been largely neglected. I suspect that most of the accessory gland products of *C. dalmanni* will fall into the latter category and a thorough investigation of the genes expressed in the accessory glands of *C. dalmanni* could provide a useful counterpoint to studies in *D. melanogaster*. A cDNA library of genes expressed uniquely in the accessory glands of *C. dalmanni* is available.

Characterisation of these sequences will provide many opportunities for determining the function of accessory gland products in *C. dalmanni*. First, function can be inferred from sequence homology with known accessory gland genes in other species. Second, mapping the chromosomal location of these sequences will allow the use of an extensive library of *C. dalmanni* microsatellites (Wright *et al.* 2004) to examine the effects of allelic variation in genes expressed in the accessory glands on components of male reproductive success. Finally, knowledge of the sequences of genes expressed in the accessory glands of *C. dalmanni* will allow the characterisation of gene function using sophisticated genetic techniques (e.g. genetic knock-down or knock-out) once these tools become available in *C. dalmanni*.

7.5.2 Genetic and physiological determinants of eyespan

In Chapter 6, I argued that juvenile hormone (JH) might play a role in the development of eyestalks and provide a physiological link between male eyespan and condition. However, a much greater understanding of the role of JH in determining eyespan is required to gain any insight into the physiological mechanisms underlying condition-dependent sexual selection. Although our current ability to apply molecular tools to research on *C. dalmanni* is limited, a number of important avenues of research should be explored.

If JH acts as a physiological link between larval condition and male eyespan, we would expect larvae (or prepupae) exhibiting high circulating levels of JH (during a particular stage in development) to metamorphose into adults with large eyespan. Larval (and adult) haemolymph JH titres can be measured using radioimmunoassay techniques (Goodman *et al.* 1995). Unfortunately, it is not possible to measure both eyespan and larval JH titre in a single individual because specimen must be sacrificed to perform radioimmunoassays. However, one could gain insight into the influence of

JH on eyestalk development by comparing larval JH profiles in males to females, dimorphic species to closely related monomorphic species, or males artificially selected for large eyespan to those artificially selected for small eyespan. These comparisons require the ability to distinguish between male and female larvae in a wide range of Diopsid species, which can be accomplished by examination of the morphology of the genital imaginal disc (Carr *et al.* in preparation). As male eyespan reflects adult components of fitness (Chapter 2), JH might also provide a physiological link between male eyespan and adult condition. Radioimmunoassays could be used to measure the haemolymph JH titres of adult males of known eyespan, and to compare the JH titres of large and small eyespan males exposed to different levels of environmental stress.

JH plays an important role in the regulation of imaginal disc cell proliferation. The sensitivity to JH of particular imaginal discs or specific regions within an imaginal disc might be regulated by the expression of proteins that interact with JH. One might expect the expression of these proteins in the region of the eye-antennal imaginal disc that gives rise to the eyestalks to be higher in males than in females. A small number of genes whose products interact with JH have been well characterized in *D. melanogaster* (e.g. *Met* and *usp*), and their expression could be investigated in *C. dalmanni* using immunohistochemical or *in situ* hybridization techniques.

JH levels are likely regulated by the insulin/TOR nutrient-sensing network (Tatar 2004). This pathway contributes to the regulation of growth, reproduction, longevity, stress resistance and metabolism in response to the availability of amino acids and carbohydrates (Nijhout 2003, Tatar 2004, Broughton *et al.* 2005, Martin & Hall 2005) and is therefore an important determinant of condition. Moreover, the insulin/TOR signalling pathway can regulate cell size and cell number in a cell-autonomous manner (Brogiolo *et al.* 2001). Genetic manipulation of insulin/TOR signalling has been used to alter the relative sizes of organs in *D. melanogaster*. For

instance, selectively removing *chico* (an insulin receptor substrate) function from the eye-antennal imaginal disc results in adult flies exhibiting disproportionately small eyes and head capsules relative to the rest of their bodies (Böhni *et al.* 1999). The importance of the insulin/TOR network to condition and its ability to alter the relative proportions of organs strongly suggest a role for genes in this network in linking condition to the expression of condition-dependent male ornaments such as male eyespan. Future studies of stalk-eyed flies should investigate the expression of insulin/TOR signalling genes in the eye-antennal imaginal disc, again comparing males to females, dimorphic species to monomorphic species, and males artificially selected for large eyespan to those selected for small eyespan.

7.5.3 Comparative studies of Diopsids

While the extremely high mating rates and ejaculate-limited reproductive success observed in *C. dalmanni* and *C. whitei* make these species ideal models for investigating the phenotype-linked fertility hypothesis, these traits seem to be unusual amongst the Diopsidae. Other species exhibit much lower mating frequencies and transfer much larger spermatophores during copulation (Kotrba 1996). Consequently, it is entirely possible that the direct fertility benefits gained through preference for large eyespan males are unimportant in other species of stalk-eyed flies. Indeed, while there are almost certainly multiple benefits associated with female preference within a given species, these benefits likely differ markedly between species. Comparative studies of the signalling function of eyespan in different Diopsid species might help to reveal these many different mechanisms and to move sexual selection research towards a more pluralistic approach to understanding the evolution of female preferences for exaggerated male secondary sexual ornaments.

Comparative studies could help elucidate the importance of reproductive organ size in stalk-eyed flies. Although I was unable to find any effects of testis size on mating frequency in *C. dalmanni*, preliminary results suggest a positive correlation between testis length and female remating rate across 7 different Diopsid species (K. Kraaijeveld, unpublished data). If testis length is adapted to lifetime mating frequency rather than short intervals of high mating frequency, such a correlation would not have been detected in the studies described in Chapters 4 and 5.

Studies of other Diopsid species might also help to address specific questions. For instance, the lack of overt rejection behaviour in female *C. dalmanni* makes detailed investigations of female choice extremely difficult. However, female *Diasemopsis meigenii* – a highly sexually dimorphic African Diopsid – sometimes violently resist male copulation attempts by rocking vigourously and dislodging the mounted male (personal observation). Preliminary investigations have revealed that the probability of observing a rejection response decreases with increasing male eyespan. Moreover, the strength of female preference is positively correlated with female eyespan (S Cotton, DW Rogers, J Small, A. Pomiankowski, and K Fowler; unpublished data). Consequently, *D. meigenii* offers many opportunities to further our knowledge of female choice in Diopsids and to test hypotheses about the origins and condition dependence of female preference.

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Appendix